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VOLUME 132

FEBRUARY TO JUNE 1967

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Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

THE BIOLOGICAL BULLETIN is issued six times a year at the Lancaster Press, Inc., Prince and Lemon Streets, Lancaster, Pennsylvania.

Subscriptions and similar matter should be addressed to The Biological Bulletin, Marine Biological Laboratory, Woods Hole, Massachusetts. Agent for Great Britain: Wheldon and Wesley, Limited, 2, 3 and 4 Arthur Street, New Oxford Street, London, W. C. 2. Single numbers, $3.75. Subscription per volume (three issues), $9.00.

Communications relative to manuscripts should be sent to Dr. Donald P. Costello, Marine Biological Laboratory, Woods Hole, Massachusetts, between June 1 and September 1, and to Dr. Donald P. Costello, P.O. Box 429, Chapel Hill, North Carolina 27514, during the remainder of the year.

Second-class postage paid at Lancaster, Pa.

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ERRATUM

In the legend to Figure 6, in the paper by Pikó, Tyler and Vinograd in the February, 1967, issue of THE BIOLOGICAL BULLETIN (volume 132, no. 1, page 81), the first sentence should read as follows: "Melting profiles of DNAs (in 0.15 *M* NaCl-0.015 *M* sodium citrate, pH 7) from *L. pictus*."

Vol. 132, No. 1 February, 1967

THE
BIOLOGICAL BULLETIN
PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

THE SUBCELLULAR DISTRIBUTIONS OF SOME HYDROLYTIC ENZYMES IN UNFERTILIZED EGGS OF THE SEA URCHIN, ARBACIA PUNCTULATA

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The identification of specific cellular granules containing hydrolytic enzymes was first made by de Duve and co-workers (1955) by centrifugal fractionation of rat-liver homogenates. The name lysosome was proposed at this time for these granules because several distinct acid hydrolases appeared to be located within them and to be released in a paralleled manner in preparations subjected to disruptive treatments such as freezing and thawing. At least 12 hydrolytic enzymes showing an acid pH optimum are presently believed to be associated with the lysosome. These include ribonuclease, acid deoxyribonuclease, acid phosphatase, phosphoprotein phosphatase, cathepsin, collagenase, *alpha*-glucosidase, *beta*-N-acetylglucosaminidase, *beta*-glucuronidase, *alpha*-mannosidase, and aryl-sulfatase (*cf.* review by Novikoff, 1961). Although most of the work on the distribution of hydrolytic enzymes has been with adult mammalian tissue, investigations of acid hydrolases have also been extended to some invertebrates. In most of these, however, only brief surveys have been made of characteristic enzymes, and no attempts to isolate any specific granules have been reported (*cf.* review by de Reuck and Cameron, 1963).

Lysosomal granules have been implicated in a few developmental processes involving regression and resorption of embryonic cells, especially in Mullerian duct rudiments of male chick embryos (Scheib-Pfleger and Wattiaux, 1962) and in the tails of amphibian tadpoles undergoing metamorphosis (Weber, 1963). In the rat egg Dalcq (1963) has observed granules in which high acid phosphatase was demonstrated cytochemically and which stained metachromatically. The metachromatic granules observed in invertebrate eggs appear to be of two types, designated as *alpha* and *beta* granules by Pasteels and Mulnard (1957), who concluded that the larger *beta* granules were not stained directly but received dye from the smaller *alpha* granules. These investigators found by centrifugation that the *alpha* granules are concentrated near the centrifugal extremity of the egg or blastomeres, while the

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[1] Submitted in partial fulfillment of the requirements for the Master of Arts degree. This work was supported by NSF grant GB-710.

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beta granules are sedimented in the hyaline part of the cytoplasm. Dalcq (1963) has recently concluded from comparative electron microscopic and cytochemical investigations of early developmental stages that the yolk platelets are a source not only of nutrient material but also of definite organelles with phosphatase activity. Pasteels and de Harven (1963) confirmed this by a series of electron microscope findings which demonstrated the transformation of yolk platelets into microvesicular bodies similar to the metachromatic granules observed in the living eggs, and the rupture of the microvesicular bodies to release minute phosphohydrolase granules. The only resemblances these granules have to lysosomes, however, are their apparent ability to rupture, and their possession of acid phosphatase.

Attempts to identify specific acid hydrolase granules in embryonic tissue have usually been made only by means of electron microscopy and cytochemistry. Dalcq (1963) proposed at a recent symposium that the most direct approach to determining the presence or absence of particles containing specific hydrolases would be the application of density gradient centrifugation to homogenates of eggs at various stages, to determine whether a layer of particles containing an array of lytic enzymes could be isolated. This present investigation is concerned with the problem of attempting to isolate a fraction containing an appreciable concentration of acid hydrolases from homogenates of unfertilized eggs of *Arbacia punctulata*. To determine the distribution within the subcellular fractions differential centrifugation has been applied to sucrose homogenates of both eggs and adult gut tissue. Isolation of specific sets of granules containing acid hydrolase activity has been attempted only on the large visible-granule fraction of the egg homogenate, by centrifugation on sucrose density gradients. The preliminary results obtained by the differential and density gradient centrifugation indicate the probable existence in the egg of more than one type of large granule possessing hydrolytic enzymes.

Materials and Methods

Biolological procedures

The animals used in this study, *Arbacia punctulata,* were collected along the Eastern Shore region of the Chesapeake Bay. They were stored in the laboratory at 23° C. in aerated, polyethylene aquaria filled with artificial sea water at 33‰ made from Utility Seven-Seas Marine Mix (Utility Chemical Company, Patterson, New Jersey). In order to obtain adult tissue, the entire gut was removed by dissection, placed in cold, artificial sea water, blotted carefully, and weighed. Eggs were obtained by electrically inducing shedding in the females, using alternating current at 30 volts (Harvey, 1954). The eggs were washed three times by settling in artificial sea water prepared from reagent grade salts and distilled water. To remove the jelly coat, the eggs were treated with acid sea water at pH 4.6–4.8 and allowed to settle (Allen, 1957). The eggs were washed an additional time in sea water buffered at pH 8.0 with 0.02 M tris-(hydroxymethyl)aminomethane (hereafter referred to as "tris") and packed in a hand centrifuge to prepare them for homogenization.

Adult gut and eggs were prepared for fractionation by the same methods. A 10% homogenate of each tissue was made, based on a weight-volume ratio for the gut tissues and on a volume/volume ratio in the eggs. The tissues were homogenized at 0° C. in a hand-operated Tenbroeck glass homogenizer in 0.98 M sucrose containing 10^{-3} M ethylenediamine tetraacetic acid (EDTA) and buffered at pH 7.5 with 0.05M tris. This buffered sucrose was used for suspending all of the cell fractions obtained by later centrifugation. A starting material which was essentially nuclei-free was prepared, based on the procedure of Berthet and de Duve (1951) with modifications to allow for the differences in sucrose density. The 10% homogenate was centrifuged for ten minutes at 2000 rpm for the gut homogenate and at 500 rpm for the egg homogenate, in rotor No. 253 in an International PR-2 centrifuge at 0° C. The egg homogenate was centrifuged at the much lower speed to remove the debris and whole cells without removing the majority of heavy granules. The supernatant fluid was then decanted and saved. The sedimented fraction from each tissue was rehomogenized in an additional 3 to 5 ml. of buffered 0.98 M sucrose-EDTA and recentrifuged at the same speed as before. The combined supernatant fluids were used for the subsequent isolation of granules. The final precipitate containing clumped nuclei, cell fragments, and whole cells was discarded.

The various subcellular fractions were obtained by a modification of the technique used by Applemans, Wattiaux and de Duve (1955). The procedure is outlined in the flow sheet given below. All of the operations described were carried out at or near 0° C. The preparations were either used immediately or frozen at −18° C. for up to 48 hours. Tests of the effects of freezing on enzyme activities were made on whole homogenates. None of the enzymes for which data are reported were decreased in activity as a result of freezing.

Fraction I from eggs was centrifuged on a layered sucrose gradient to separate particles of different densities (de Duve, Berthet and Beaufay, 1959). The separated granule layers were removed by pipetting from the top or by puncturing the bottom of the tube and allowing the sucrose to drip out slowly.

The staining properties of the granules obtained on the density gradient were examined by dividing Fraction I into three equal portions. One tube served as a control; the other two contained 10 to 15 drops of 0.1% dye in a total volume of 5 ml. After an initial centrifugation at 17,500 times gravity to remove the excess stain, the stained preparations were placed over the same density gradients as above, and centrifuged for one hour at 90,000 times gravity.

Chemical procedures

Determinations of protein, nucleic acid, acid phosphatase, esterase, lipase, arylsulfatase, *beta*-galactosidase, ribonuclease (RNAase), and proteolytic activity were attempted according to the methods outlined below. All enzyme reactions were run at 25° C. Tests for linearity were made on whole homogenates in preliminary experiments.

Before determinations of protein and nucleic acid were made, the samples were extracted according to the method of Schmidt and Thannhauser (1945) three times with cold 10% W/V trichloroacetic acid (TCA), twice with boiling ethanol-ether (3:1 V/V) and twice with hot 5% TCA at 90° for 15 minutes. The hot TCA extracts were combined and used for nucleic acid determination by the ultraviolet absorption procedure of Schneider (1957). The protein was suspended in 1 N sodium hydroxide and determined with Folin-Ciocalteau reagent (Fisher Chemical

Company) by the method of Lowry *et al.* (1951). Standard absorbance curves were prepared, using solutions of reagent grade RNA and of crystalline bovine serum albumin, both obtained from Nutritional Biochemicals Corporation.

Assays of acid phosphatase, esterase, lipase, aryl-sulfatase and *beta*-galactosidase were attempted by using as substrates *p*-nitrophenyl phosphate, acetate, stearate, sulfate and galactoside, respectively. All substrates were obtained from Nutritional Biochemicals Corporation, except for *p*-nitrophenyl phosphate, and *p*-nitrophenyl-β-D galactoside, which were obtained from the Sigma Chemical Company. The *p*-nitrophenol liberated from each substrate was determined in alkaline solution (except as noted below) at 400 mμ on the Beckman DU spectrophotometer, or with filter No. 42 on the Klett-Summerson colorimeter. The details of each procedure are presented below.

Acid phosphatase was determined by the method of Burch *et al.* (1952). Esterase was assayed at pH 7.1 by the method of Huggins and Laprides (1947), using a standard curve of *p*-nitrophenol at the same pH. The method used for aryl-sulfatase depends on the rather small change in absorbance at 400 mμ which occurs when *p*-nitrophenyl sulfate and enzyme are incubated at pH 5.1. The reaction mixture contained 2.0 mg. of substrate, 0.03 M sodium acetate, pH 5.0 and 0.05 to 0.15 ml. of enzyme in a total volume of 3.0 ml. Readings were taken for 15 minutes on the DU or DB spectrophotometer against a blank containing buffer and enzyme. Spontaneous hydrolysis of the substrate was negligible at this pH. A standard curve was prepared from *p*-nitrophenol at pH 5.1. Ribonuclease was measured by the method of Dubos and Thompson (1938).

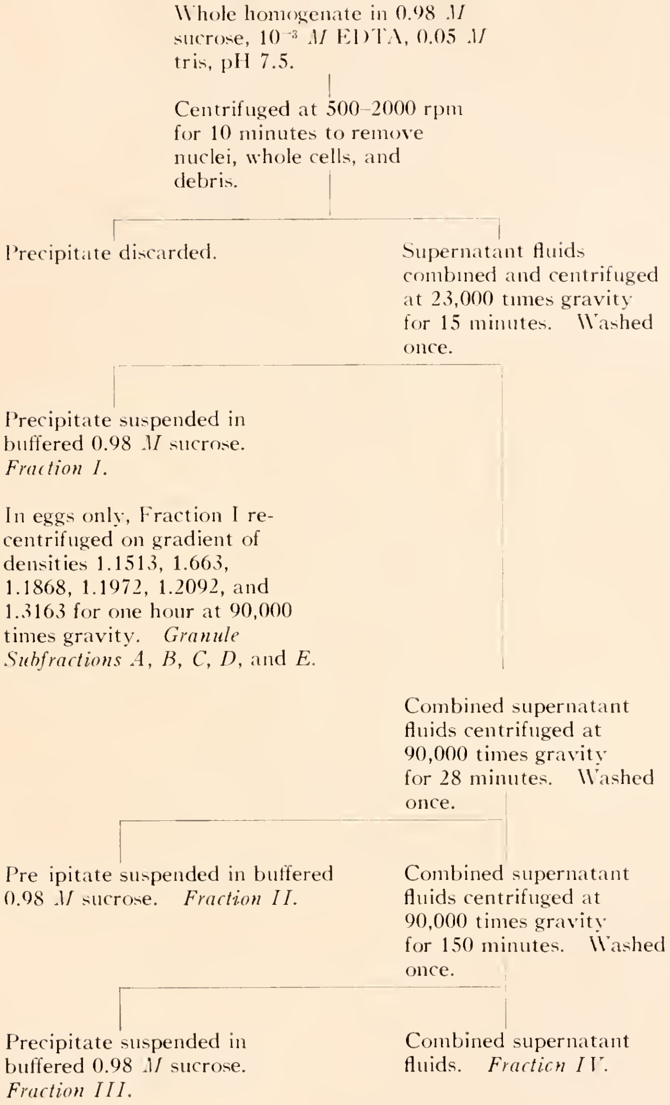
Attempts to measure protease activity at pH 5 were made by the method of Anson (1938), using the liberation of tyrosine from denatured hemoglobin. This method produced extremely variable results with both whole homogenates and subcellular fractions. Whole homogenates gave high blank values, presumably because of a high content of free tyrosine. The assay of *beta*-galactosidase was attempted by the method of Wallenfels (1962), using *p*-nitrophenyl-*beta*-galactoside. No activity could be detected in the whole homogenates, and assays were not performed on subcellular fractions. The assays for lipase, using *p*-nitrophenyl stearate (Huggins and Laprides, 1947), were also negative in whole homogenates of eggs and gut. Attempts to measure succinic dehydrogenase by the ferricyanide reduction method of Bonner (1955) were made only on fresh preparations of Fraction I and its subfractions. This reaction was complicated by the large amount of pigment in this fraction, and it was decided that the data are probably unreliable.

Results

Distribution of enzymes and nucleic acid in subcellular fractions

The distributions of acid phosphatase, esterase, and nucleic acid in the various fractions obtained by differential centrifugation of the gut are shown in Table I. The highest total acid phosphatase and esterase activities are found in the soluble fraction, while the large granules contain only 10 to 15% of the total activity. The microsomal fraction contains the highest specific activity of esterase while in most experiments the specific activity of acid phosphatase was found to be rather uniformly higher in all the granule fractions than in the whole homogenate. The

Outline of Fractionation Procedure



Whole homogenate in 0.98 *M* sucrose, 10^{-3} *M* EDTA, 0.05 *M* tris, pH 7.5.

Centrifuged at 500–2000 rpm for 10 minutes to remove nuclei, whole cells, and debris.

Precipitate discarded.

Supernatant fluids combined and centrifuged at 23,000 times gravity for 15 minutes. Washed once.

Precipitate suspended in buffered 0.98 *M* sucrose. *Fraction I.*

In eggs only, Fraction I recentrifuged on gradient of densities 1.1513, 1.663, 1.1868, 1.1972, 1.2092, and 1.3163 for one hour at 90,000 times gravity. *Granule Subfractions A, B, C, D,* and *E.*

Combined supernatant fluids centrifuged at 90,000 times gravity for 28 minutes. Washed once.

Precipitate suspended in buffered 0.98 *M* sucrose. *Fraction II.*

Combined supernatant fluids centrifuged at 90,000 times gravity for 150 minutes. Washed once.

Precipitate suspended in buffered 0.98 *M* sucrose. *Fraction III.*

Combined supernatant fluids. *Fraction IV.*

highest per cent of total nucleic acid, as determined by the ultraviolet method, is present in the supernatant fraction, representing very likely mostly soluble ribonucleic acid. The percentage in the microsomal fraction, although slightly higher than in the larger granules, is still quite low, possibly indicating that the cells are poor in ribosomal ribonucleic acid. These findings may be a consequence of the starvation of the animals prior to the fractionation of the tissue.

The distributions of enzyme activities within the subcellular fractions of the eggs are presented in Table II. In addition to the enzymes examined in the gut

TABLE I

Distribution of enzyme activities, nucleic acid and protein in subcellular fractions of adult gut

Specific activity = μM substrate converted/min./mg. protein = factor indicated. Standard errors are given. Number of experiments is indicated after each title.

	Whole	I	II	III	IV	Total %
Acid phosphatase (4)						
Per cent	100	15.5±1.1	8.9±2.0	8.2± 2.6	40.8± 4.3	73.4± 3.2
Sp. act. $\times 10^5$	6.0±0.8	11.9±1.5	11.9±2.0	12.3± 1.2	3.9± 1.2	
Esterase (2)						
Per cent	100	9.3±2.0	8.7±0.0	22.6±11.0	97.6± 0.6	138.2±12.0
Sp. act. $\times 10^5$	1.4±0.0	1.5±0.0	2.0±0.9	4.4± 0.4	1.6± 0.0	
Nucleic acid (2)						
Per cent	100	8.7±2.4	5.2±1.3	10.2± 2.0	70.0± 5.7	94.1±0.2
Sp. amt. $\times 10^2$	2.2±0.0	2.1±0.0	1.4±0.1	2.6± 0.6	1.8± 0.4	
Protein (4)						
Per cent	100	8.2±0.4	4.9±1.7	4.7± 2.2	70.0±11.2	87.8±15.0

subcellular fractions, determinations were made of aryl-sulfatase and RNAase. The highest total acid phosphatase and esterase activities are present in the soluble fractions, but the large granules contain from 25 to 50% of the total acid phosphatase activity and from 15 to 40% of the esterase activity. This difference in percentage of activity present in the large granules of the eggs is probably due to the presence of granules which are not present in the gut. The highest specific activity of esterase is found in the microsomal fraction (III) ; in this respect the distribution

TABLE II

Distribution of enzyme activities, nucleic acid and protein in subcellular fractions of the egg

Specific activity for RNAase is expressed as μg RNA solubilized/min./μg protein. All other activities are as in Table I.

	Whole	I	II	III	IV	Total %
Acid phosphatase (4)						
Per cent	100	36.1±5.2	11.3±2.9	5.8±0.9	62.3± 7.9	115.5±7.6
Sp. act. $\times 10^5$	3.5±0.4	7.1±0.6	6.8±2.0	13.2±3.9	2.5± 0.5	
Esterase (4)						
Per cent	100	23.2±6.4	12.3±2.8	5.9±0.7	69.2± 3.7	110.6± 7.2
Sp. act. $\times 10^6$	8.3±1.1	11.2±3.3	18.2±3.6	42.3±7.6	9.4± 2.1	
Aryl-sulfatase (3)						
Per cent	100	26.4±3.5	2.1±0.5	0.9±0.4	33.5± 7.2	62.9± 9.0
Sp. act. $\times 10^4$	2.4±0.6	1.9±0.6	0.5±0.1	0.5±0.3	1.4± 0	
RNAase (3)						
Per cent	100	32.1±2.3	17.7±0.5	15.5±1.4	83.6± 7.7	148.9± 9.0
Sp. act. $\times 10^2$	4.7±0.8	4.5±0.8	8.0±2.9	9.8±2.4	8.8± 1.3	
Nucleic acid (3)						
Per cent	100	20.3±1.4	14.2±3.2	32.8±7.0	65.6±12.0	132.9±34.3
Sp. amt. $\times 10^2$	7.7±0.2	4.7±0.7	10.4±0.8	34.4±5.0	11.2± 2.3	
Protein (7)						
Per cent	100	24.3±3.5	7.7±1.2	4.1±1.2	60.3± 6.7	96.4±15.5

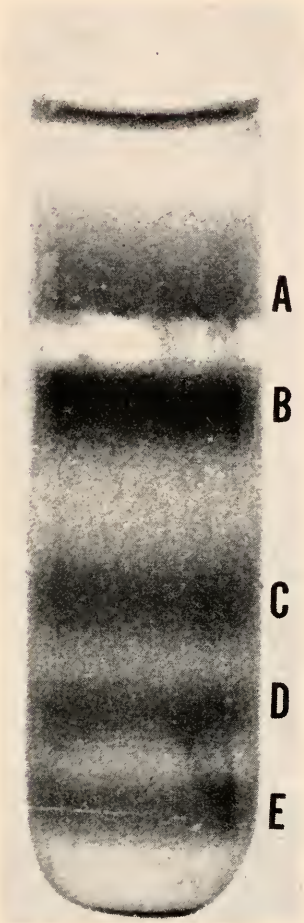


FIGURE 1. Subfractions obtained from Fraction I by density gradient centrifugation. The subfractions were obtained by centrifuging granules on the following densities of sucrose for one hour at 90,000 times gravity: 1.1513, 1.1663, 1.1868, 1.1972, 1.2092, and 1.3163.

is similar to that in the adult intestine. The acid phosphatase in the egg also resembles that of the gut in being rather consistently more concentrated in all granule fractions than in the whole homogenate. The distribution of aryl-sulfatase ranges from 20 to 30% in Fraction I and from 20 to 40% in Fraction IV. The highest specific activity of RNAase, as well as the greatest nucleic acid: protein ratios, were found in the microsomal fraction, Fraction III. The highest percentages of both RNAase activity and total nucleic acid are found in the soluble fraction; however, the recoveries of both these substances are quite high, when the combined amounts in the separated fractions are compared to those in the whole homogenates. It is interesting to note that considerable percentages of the recovered nucleic acid and RNAase were found to be present in the visible granule fraction (I), and in the intermediate granules (II), indicating the probable association of both substances with non-microsomal particles. This association is examined in more detail below.

Density gradient centrifugation of Fraction I

In order to investigate the possible heterogeneity of the visible granules with respect to their contents of hydrolytic enzymes, Fraction I was further centrifuged in tubes containing several layers of sucrose solutions having different densities. The separation of granules into layers of different densities as a result of this centrifugation is depicted in Figure 1. In most experiments four separable layers were obtained; in one case a fifth, denser layer was also found. Because the centrifugation was performed for only one hour, it seems unlikely that complete separation of granules of different densities was achieved. This incompleteness of separation, as well as a certain amount of mixing which occurred upon removal of the different fractions, undoubtedly contributed to the variations in distribution and activities reported below.

Assays of the hydrolytic enzymes in question, and of nucleic acid were performed on the subfractions obtained by the gradient centrifugation. Tests for DNA

TABLE III

Distribution of enzyme activities, nucleic acid and protein in subfractions derived from fraction I

Specific activities are as in Tables I and II. The letters A through F represent subtractions of different densities obtained by centrifuging Fraction I on a sucrose layer gradient. Subfraction A has the lowest density.

	A	B	C	D	E or F	Total %
Acid phosphatase (3)						
Per cent of Fr. I.	28.0± 0.0	52.3±1.3	12.7±0.4	6.1±0.6		99.1± 1.3
Sp. act. × 10⁴	3.4± 0.1	1.1±0.0	0.5±0.0	0.5±0.0		
Acid phosphatase* (2)						
Per cent of Fr. I	51.0±10.0	9.2±2.1	3.2±2.2	3.6±0.3	3.2±0.8 (F)*	70.2± 8.0
Sp. act. × 10⁴	0.5± 0.0	0.7±0.1	0.1±0.0	0.3±0.1	2.2±0.2 (F)*	
Esterase (3)						
Per cent of Fr. I.	8.6± 0.1	31.4±0.3	17.0±0.0	1.2±0.6		58.2± 1.0
Sp. act. × 10⁵	3.3± 0.1	1.9±0.0	2.1±0.1	0.2±0.1		
Aryl-sulfatase (4)						
Per cent of Fr. I	24.6± 5.2	39.8±5.9	17.8±3.3	15.9±4.6	25.1 (E)**	104.4±15.2
Sp. act. × 10⁴	3.0± 0.7	1.7±0.2	2.2±0.4	3.6±0.7	7.2 (E)**	
RNAase (3)						
Per cent of Fr. I	38.8± 3.5	39.8±6.5	35.0±4.0	33.7±2.9	41.8 (E)**	161.3±10.0
Sp. act. × 10²	8.7± 0.5	3.2±0.8	16.2±4.6	12.0±1.4	26.0 (E)	
Nucleic Acid (4)						
Per cent of Fr. I	13.2+ 1.5	23.7±4.5	18.0±4.2	14.9±3.6	15.9 (E)**	73.3±9.1
Sp. amt. × 10²	9.1± 1.5	3.8±0.5	8.8±1.8	16.5±4.8	30.0 (E)**	
Protein (8)						
Per cent of W.H.	5.3± 1.4	13.6±1.8	4.7±0.6	2.9±0.5	1.6 (E)**	27.9±2.2
Protein (2)*						
Per cent of W.H.	13.8± 2.4	2.0±0.1	5.9±0.5	1.5±0.3	0.2 (F)*	23.4± 2.2

* Eggs were washed in calcium-free water and Fraction I was centrifuged on the gradient for 90 minutes. Echinochrome granules were concentrated in Layer F.

** Layer E was obtained in only one experiment.

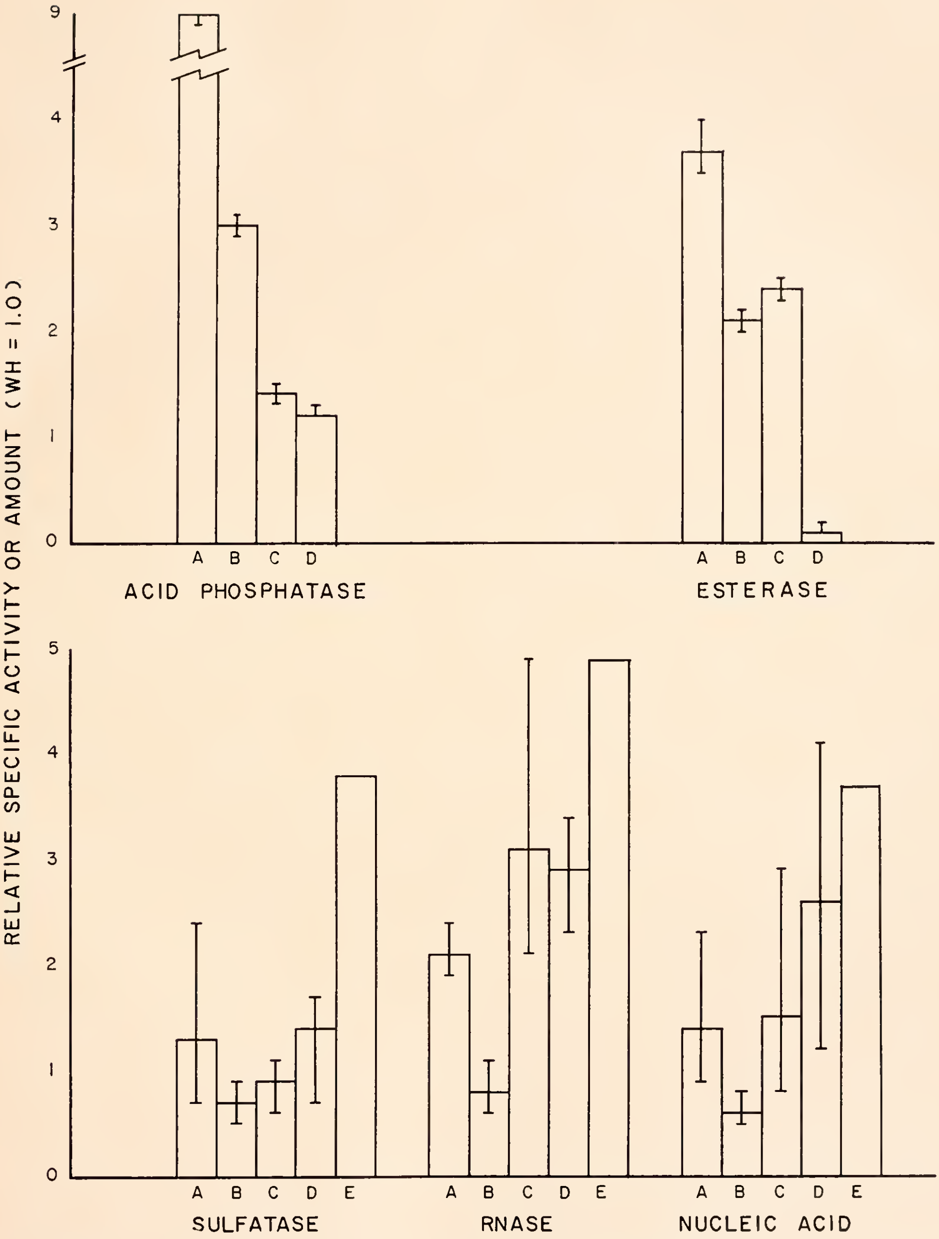


FIGURE 2. Average relative specific activities of enzymes and amounts of nucleic acid in the subfraction granules isolated from Fraction I. The specific activity of the whole homogenate is set at 1.0 for the calculations. The lines represent the range of values for three or four experiments.

were made by the diphenylamine procedure of Dische (1930) as modified by Seibert (1940). These indicated that only trace amounts were present in each fraction. The absorbance of the hot TCA extracts at 260 mμ is therefore tentatively assumed to be a measure of ribonucleic acid content. The results of these determinations are presented in Table III, and the average specific activities of enzymes and amounts of nucleic acid in each granule fraction are shown in a series of histograms in Figure 2. The results indicate considerable heterogeneity in granule types. Acid phosphatase and esterase have the highest specific activities in the granules of lowest density, Subfraction A. Acid phophatase in these granules has a relative specific activity as much as nine times that of the whole homogenate, while esterase is about four times as concentrated as in the whole homogenate. Subfraction B contains the highest percentages of most of the enzymes assayed as well as the highest percentage of total protein. This is assumed to be a result of incomplete separation of granules as noted above. The echinochrome pigment granules are also concentrated in this subfraction in most of the experiments; however, see below. Pigment presumably derived from these granules was always found in the fluid at the top of the density gradient. Aryl-sulfatase, a characteristic lysosomal enzyme, has somewhat higher specific activities in Subfractions A and in the denser granules D and E; however, its distribution is fairly uniform throughout all the subfractions isolated. Ribonuclease activity and nucleic acid are only moderately high in Subfraction A; however, the denser granules, especially D and E, contain unusually high concentrations of both RNAase and nucleic acid.

In two experiments by one of us (R. B.) the eggs were washed several times in calcium-free sea water before homogenization, and Fraction I was centrifuged on the gradient for 90 minutes instead of 60, which resulted in a difference in the distribution of both echinochrome granules and of acid phosphatase. In these experiments the echinochrome granules did not release any pigment, but were driven intact through all the density layers to the bottom of the tube. The granules were evidently in osmotic equilibrium with the 2.5 *M* sucrose, since they immediately ruptured on resuspension in the 0.98 *M* buffered sucrose. The distribution of acid phosphatase in these experiments is also indicated in Table III, with the echinochrome granule layer designated as "F." In these experiments the highest specific activity of acid phosphatase was found to be associated with the echinochrome granules, although the assays were complicated by the presence of the pigment. The echinochrome granule fraction contains a very small percentage of the total protein of Fraction I. The highest percentage of total protein and acid phosphatase was found in Subfraction A in these experiments, while very little protein was present in the B layer. These results appear to indicate that there are at least two types of large, acid phosphatase-containing particles, one of which may be the echinochrome granule.

One of the properties used to define the lysosome is the latency of enzymes in the intact particle (De Duve, 1963). It was of interest to determine whether the activities of acid phosphatase and esterase would be affected if the granules of Subfraction A were subjected to different osmotic conditions. It must be noted that under normal conditions of assay the granules would be ruptured in the dilute reaction mixture. In order to test the effect of osmotic shock, granules of this Subfraction were divided into two aliquots. The first was suspended in 0.05 *M* tris-10^{-3} M EDTA without sucrose, while the other was suspended in buffered sucrose. Assays of acid phosphatase and esterase were performed in the usual manner with the first aliquot, while for the second, all reagents were made up in buffered 0.98 M sucrose in an attempt to maintain the granules in an intact state during the reaction. The activity of acid phosphatase was increased 75% by the rupture of the granules, while esterase activity remained unchanged. Treatment with the dilute buffer caused an immediate clarification of the granule suspension. Centrifugation of the clarified suspension at 90,000 g for one hour sedimented approximately one-third of the phosphatase and two-thirds of the esterase activity. These results are somewhat inconclusive, since it is obvious that the enzymes of the intact granules are not completely latent. One possibility is that some of the granules are damaged in preparation; however, it is apparent that they still possess a semipermeable membrane. A second possibility is that the enzymes are really not latent within the granules.

Microscopical observation and vital staining of granules

The granules in Subfraction A were observed under oil immersion in order to estimate their size range. The spherical granules varied in size from about one to three microns, with 60–70% being in the 1–1½ micron range, and about 20% in the 2–2½ micron range. Only a very few granules were evident in the 3–3½ micron range, probably less than one or two per cent. No size estimates are available for granules of the heavier subfractions.

The staining of granules from Fraction I, prior to isolation on the sucrose layers, gave conclusive results only with toluidine blue. With this stain Subfractions A and E stained slightly, exhibiting a pale green color. Subfractions B, C and D appeared to exhibit metachromasia to a considerable degree, with most of the red color concentrated in Subfraction B. Neutral red and methyl red were also predominantly taken up by Subfraction B. The presence of red echinochrome pigment granules in this fraction interfered with the detection of its staining properties with all dyes. It seems clear that the granules of Subfraction A, which possess the highest specific activity of acid phosphatase, do not stain metachromatically *in vitro*. A direct analysis of the nature and content of polysaccharides in the different granules would appear to be desirable from the standpoint of correlating this finding with that of Dalcq (1963), who reported that granules possessing acid phosphatase activity also exhibited metachromatic properties.

Discussion

From the results illustrated in Figure 2, it may be tentatively concluded that at least two types of visible granules, differing in their content of hydrolytic enzymes, exist in the egg. The first type, exemplified by Subfraction A, exhibits acid phosphatase and esterase activities. The second type, found in Subfractions D and E, contains RNAase and nucleic acid, as well as a relatively high content of arylsulfatase.

The granules of Subfraction A contain at least two of the hydrolytic enzymes believed by de Duve (1963), Novikoff (1961) and others to be located within the lysosomes. The absence of metachromasia in these granules after *in vitro* staining suggests that they may differ from the *alpha* and *beta* granules of Dalcq (1963) and Pasteels and Mulnard (1957) in their polysaccharide content; however, these investigators worked only with fertilized eggs stained *in vivo*. Rebhun (1959) demonstrated that in *Spisula solidissima* staining of the *alpha* and *beta* granules appeared only after fertilization. In stratifying eggs of various species of sea urchins, Immers (1960) expressed doubt that regions of mucopolysaccharide concentration evident after *in vivo* staining corresponded to the metachromatic *alpha* and *beta* granules of Dalcq and Pasteels because his staining was performed only on unfertilized eggs.

In spite of their high content of hydrolytic enzymes, it would be premature to identify the granules of Subfraction A as lysosomes. De Duve (1963) has warned that the present definition of the lysosome, although based primarily on his rat liver tissue work, must not include any incidental details such as size and other physical characters, osmotic properties, centrifugal behavior, mechanism of structure-linked latency, or sensitivity to individual disrupting treatments. If these factors are therefore omitted in defining the lysosome, the essential characteristic remaining is the association within a special group of cytoplasmic particles of a number of soluble acid hydrolases of widely differing specificity. The accessibility of these enzymes to the surrounding substrate must be restricted, making the latency of the enzymes dependent on the structural complexes of the particles. Such a definition would be broad enough to include the hydrolytic granules in Subfraction A isolated from *Arbacia* eggs, if it could be shown more conclusively that the accesssibility or activity of the enzymes in question is restricted by the granular structure.

The finding that acid phosphatase activity is associated with the echinochrome pigment granules is of considerable interest. Since these granules were ruptured by dilution after recovery from the 2.5 *M* sucrose layer, the effects of different osmotic treatments were not tested. Further experiments on these granules are in progress.

We believe that the mitochondria are concentrated in Subfraction B, since it contains the highest percentage and specific activity of succinic dehydrogenase; however, the data for this enzyme appear to be rather unreliable. This subfraction is probably heterogeneous, since it contains the highest percentages of all enzymes and of total protein.

The most dense granules in the visible granule fraction, recovered in Subfractions D and E after gradient centrifugation, contain much higher specific concentrations of nucleic acid and RNAase than do the other visible granules. In preliminary experiments we have made determinations of amino acid incorporation into protein of these heavy granules after giving unfertilized eggs a 10-minute pulse with C^{14}-phenylalanine. After such a pulse, the specific activity of Subfractions D and E, calculated on the basis of nucleic acid content, is only about one-tenth that of the microsome fraction; these granules are therefore quite inactive in protein synthesis, even in the fertilized egg. We have concluded that there is little contamination from microsomes in this fraction. The existence of dense RNA bodies in eggs has been reported by other workers. Raven (1945) demonstrated the presence of heavy RNA particles in the centrifugal pole of stratified *Limnaea* eggs. Pasteels (1958), by centrifuging *Paracentrotus* eggs, discovered "heavy bodies" of

RNA, ranging from 1–3 microns, in the centrifugal cap region. This region, which also contained the mitochondria, was intensely stained with pyronine. Pasteels postulated that, in addition to being found in the ribosomes and in annulate membranes within the egg, RNA could also be found in undefined structures that could be linked to the mitochondria but which contained the most dense material in the egg. Balinsky and Devis (1963) observed electron-dense granules in the young oocytes of *Xenopus laevis* which presumably accumulated between adjacent mitochondria. Afzelius (1956) has also described "heavy bodies" which stain vitally with toluidine blue in the sea urchin egg. Immers (1960) described dense RNA granules which were separate from the mitochondria in the most centrifugal zone of stratified eggs of *Paracentrotus lividus*. A few workers have claimed that the heavy yolk granules, especially in the Amphibia, contain an appreciable amount of RNA (Grant, 1953; Rounds and Flickinger, 1958), but others have shown by histochemical and cytological studies that there is little or no RNA within the yolk granules of most species examined. Collier (1960) found no evidence of either RNA or proteolytic enzymes in the yolk granules of *Ilyanassa obsoleta*. The recent work by Karasaki (1963) and Ohno *et al.* (1963) revealed no evidence for the presence of RNA in the yolk granules of *Triturus pyrrhogaster* and *Rana pipiens* embryos.

It may be tentatively concluded that the heavy granules in Subfractions D and E correspond to those described by Immers and Pasteels. It is possible that the annulate lamellae described by Pasteels (1958) are sufficiently dense to be included in this fraction; these structures consist of membranes to which bodies similar in size and density to ribosomes are attached. No previous report has been made concerning the association of RNAase with any large granule fraction in the egg; however, a comparison of the present finding with that of Reid and Node (1959) for granules of rat liver is of particular interest. These authors provided evidence that acid RNAase was present in particles which were more rapidly sedimented from homogenates than the lysosomes, indicating the possible existence of a separate set of granules which contain this enzyme.

The distribution of activities of the hydrolytic enzymes in Fractions II and III of the egg homogenates indicates the presence of these enzymes in submicroscopic structures (see Table II). The possibility therefore exists that granules resembling rat liver lysosomes in size may also be present in the egg. If such granules are easily ruptured during preparation, as are liver lysosomes, this may account for the high enzyme activities found in the soluble fraction. It is of interest that Fractions II and III of the gut tissue of adult *Arbacia* (Table I) contain approximately the same specific activities of acid phosphatase and esterase as do the corresponding fractions of the egg.

The heterogeneity of the populations of granules which contain hydrolytic enzymes in the egg may be generally related to the timing with which different enzymes become active during development. It is postulated that such a separation of enzymes in different granules could result in the specific release or activation of some hydrolases, but not others, at particular developmental stages. Furthermore, partial segregation of the granules into different cells during cleavage may confer different developmental potentialities on the daughter cells. Segregation of granules and certain enzymes have been observed in numerous eggs exhibiting "mosaic" cleavage (*cf.* Brachet, 1950, for review); however, similar differentiation has not been observed in the sea urchin. Experimental testing of the latter hypothesis must await the development of techniques for visual identification of the granules in question.

Summary

Differential centrifugation and density gradient centrifugation have been applied to nuclei-free homogenates of unfertilized eggs and adult gut of *Arbacia*, to determine the distributions of several hydrolytic enzymes and of nucleic acid and protein. Two types of large visible granules have been partially separated from egg homogenates by gradient centrifugation. The first type is rich in acid phosphatase and esterase; the second contains sulfatase, RNAase and nucleic acid. The activities of the above enzymes have also been determined in microsomal and soluble fractions of the egg, and the distribution of acid phosphatase and esterase have also been determined in the major subcellular fractions of the adult gut of *Arbacia*. In both types of homogenates hydrolytic enzymes were found to be present in submicroscopic granules and in the supernatant fluid. The major difference in the two types of material is that large granules containing the enzymes are present in the eggs but not in the gut tissue.

LITERATURE CITED

Afzelius, B. A., 1956. The ultrastructure of the cortical granules and their products in the sea urchin egg as studied with the electron microscope. *Exp. Cell Res.*, **10**: 257–285.

Allen, R. D., 1957. Isolation of cortical material from sea urchin eggs by centrifugation. *J. Cell. Comp. Physiol.*, **49**: 379–394.

Anson, M. L., 1938. The estimation of pepsin, trypsin, papain and cathepsin with hemoglobin. *J. Gen. Physiol.*, **22**: 79–91.

Applemans, F., R. Wattiaux and C. de Duve, 1955. Tissue fractionation studies. 5. The association of acid phosphatase with a special class of cytoplasmic granules in rat liver. *Biochem. J.*, **59**: 438–445.

Balinsky, B. I., and R. J. Devis, 1963. Origin and differentiation of cytoplasmic structures in the oocytes of *Xenopus laevis*. *Acta Embry. Morph. Exp.*, **6**: 55–108.

Berthet, J., and C. de Duve, 1951. Tissue fractionation studies. 1. The existence of a mitochondria linked, enzymically inactive form of acid phosphatase in rat-liver tissue. *Biochem. J.*, **50**: 174–181.

Bonner, W. D., 1955. Succinic dehydrogenase. *In*: Methods in Enzymology, Vol. III, S. P. Colowick and N. O. Kaplan, eds., Academic Press, Inc., New York.

Brachet, J., 1950. Chemical Embryology. Interscience Publishers, Inc., New York.

Burch, H. B., O. A. Bessey, R. H. Love and O. H. Lowry, 1952. The determination of thiamine and thiamine phosphates in small quantities of blood and blood cells. *J. Biol. Chem.*, **198**: 477–490.

Collier, J. R., 1960. The localization of ribonucleic acid in the eggs of *Ilyanassa obsoleta*. *Exp. Cell. Res.*, **21**: 126–136.

Dalcq, A. M., 1963. The relation to lysosomes of the *in vivo* metachromatic granules. *In*: Ciba Foundation Symposium on Lysosomes, A. V. S. de Reuck and M. P. Cameron, eds., Little, Brown and Co., Boston.

de Duve, C., 1963. The lysosome concept. *In*: Ciba Foundation Symposium on Lysosomes, A. V. S. de Reuck and M. P. Cameron, eds., Little, Brown and Co., Boston.

de Duve, C., J. Berthet, and H. Beaufay, 1959. Gradient centrifugation of cell particles: theory and applications. *Prog. Biophysics and Biophysical Chem.*, **9**: 325–369.

de Duve, C., B. C. Pressman, R. Gianetto, R. Wattiaux and F. Appelmans, 1955. Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat-liver tissue. *Biochem. J.*, **60**: 604–617.

de Reuck, A. V. S., and M. P. Cameron, eds., 1963. Ciba Foundation Symposium on Lysosomes. Little, Brown and Co., Boston.

Dubos, R. J., and R. H. S. Thompson, 1938. The decomposition of yeast nucleic acid by a heat-resistant enzyme. *J. Biol. Chem.,* **124**: 501–510.

Dische, Z., 1930. Some new characteristic color tests for thymonucleic acid and a microchemical method for determining the same in animal organs by means of this test. *Mikrochemie,* **8**: 4–32.

Grant, P., 1953. Phosphate metabolism during oogenesis in *Rana temporaria. J. Exp. Zool.,* **124**: 513–543.

Harvey, E. B., 1954. Electrical method of determining the sex of sea urchins. *Nature,* **173**: 86.

Huggins, C., and J. Laprides, 1947. Chromogenic substrates. IV. Acyl esters of *p*-nitrophenol as substrates for the colorimetric determination of esterase. *J. Biol. Chem.,* **170**: 467–482.

Immers, J., 1960. Studies on cytoplasmic components of sea urchin eggs stratified by centrifugation. *Exp. Cell Res.,* **19**: 499–514.

Karasaki, S., 1963. Studies on amphibian yolk. 5. Electron microscopic observation on the utilization of yolk platelets during embryogenesis. *J. Ultrastructure Res.,* **9**: 225–247.

Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall, 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.,* **193**: 265–275.

Novikoff, A. B., 1961. Lysosomes and related particles. *In*: The Cell, Vol. II, J. Brachet and A. E. Mirsky, eds., Academic Press, Inc., New York.

Ohno, S., S. Karasaki and K. Takata, 1963. Histo- and cytochemical studies on the superficial layer of yolk platelets in the *Triturus* embryo. *Exp. Cell Res.,* **33**: 310–318.

Pasteels, J. J., 1958. Comparative cytochemistry of the fertilized egg. *In*: A Symposium on the Chemical Basis of Development. W. D. McElroy and B. Glass, eds., Johns Hopkins Press, Baltimore.

Pasteels, J. J., and E. de Harven, 1963. Study by electron microscopy of the cytoplasm of the fertilized and unfertilized egg of *Barnea candida. Arch Biol. (Liege),* **74**: 417–437.

Pasteels, J. J., and J. Mulnard, 1957. Toluidine blue in metachromasia *in vivo* and its cytochemical analysis in eggs of *B. candida, G. angulata,* and *P. miliarius. Arch. Biol. (Liege),* **68**: 115–163.

Pasteels, J. J., and J. Mulnard, 1963. The relations between *alpha* and *beta* meta-granules in the egg of the bivalve mollusk *Barnea candida. Arch. Biol. (Liege),* **74**: 319–340.

Raven, Chr. P., 1945. The development of the egg of *Limnaea stagnalis* from oviposition till first cleavage. *Arch. Néerl. Zool.,* **7**: 91–119.

Rebhun, L. I., 1959. Studies of early cleavage in the surf clam, *Spisula solidissima,* using methylene blue and toluidine blue as vital stains. *Biol. Bull.,* **117**: 518–545.

Reid, E., and J. T. Node, 1959. Liver ribonucleases. *Ann. N. Y. Acad. Sci.,* **81**: 619.

Rounds, D. E., and R. A. Flickinger, 1958. Distribution of ribonucleoprotein during neural induction in the frog embryo. *J. Exp. Zool.,* **137**: 479–499.

Scheib-Pfleger, D., and R. Wattiaux, 1962. Étude des hydrolases acides des canaux de Muller de l'embryon de Poulet. 1. Activités totales et solubles des canaux d'embryons de 8 à 10 jours d'incubation. *Dev. Biol.,* **5**: 205–217.

Schmidt, G., and S. J. Thannhauser, 1945. A method for the determination of desoxyribonucleic acid, ribonucleic acid and phosphoproteins in animal tissues. *J. Biol. Chem.,* **161**: 83–89.

Schneider, W. C., 1957. Determination of nucleic acids in tissues by pentose analysis. *In*: Methods in Enzymology, Vol. III, S. P. Colowick and N. O. Kaplan, eds., Academic Press, Inc., New York.

Seibert, F. B., 1940. Removal of the impurities, nucleic acid, and polysaccharide from tuberculin protein. *J. Biol. Chem.,* **133**: 593.

Wallenfels, K., 1962. *Beta*-galactosidase (crystalline). *In*: Methods in Enzymology, Vol. V., S. P. Colowick and N. O. Kaplan, eds., Academic Press, New York.

Weber, R., 1963. Behaviour and properties of acid hydrolases in regressing tails of tadpoles during spontaneous and induced metamorphosis *in vitro. In*: Ciba Foundation Symposium on Lysosomes, A. V. S. de Reuck and M. P. Cameron, eds., Little, Brown and Co., Boston.

OXYGEN UPTAKE AND RESPONSES TO RESPIRATORY STRESS IN SEA URCHINS[1]

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In discussions of oxygen consumption in invertebrates it is common practice to classify animals as oxygen conformers or regulators. The conformers are often referred to as being respiratory-dependent whereas the regulators are independent. Such classifications imply that the animals either vary their oxygen uptake relative to the oxygen availability in the external medium, or they show some degree of regulation by maintaining oxygen uptake in spite of a decrease in the surrounding oxygen availability. An animal showing respiratory independence usually becomes respiratory dependent as the external oxygen tension (PiO_2) decreases. The PiO_2 at which this occurs is referred to as the critical oxygen tension. The concept of respiratory dependence implies that the animal is at the mercy of the existing environmental conditions. Although such classifications may serve to clarify similarities between various animals, they may obscure basic characteristics of animal energy economy and of important factors affecting interaction of the organism and its environment. We contend in opposition to current teaching that an animal's oxygen uptake must have a closer correlation with internal oxygen tensions than with the external oxygen availability. Recent advances in gas analyzing techniques have simplified the measurement of internal oxygen tensions. The present report is based on measurements of oxygen uptake ($\dot{V}O_2$) of three species of sea urchins. Internal as well as external oxygen tensions were monitored as the animals were subjected to a variety of external conditions.

MATERIALS

Two of the three species of the genus *Strongylocentrotus* used in these experiments are extremely abundant members of the marine fauna in the vicinity of the marine laboratory at Friday Harbor, Washington. *S. droebachiensis* and *S. franciscanus* exhibit a marked subtidal zonation, with the former usually occupying the lower intertidal and the upper subtidal zones, while the latter is generally found several meters below zero tide. However, both species have been collected from depths as great as 35 meters and as intertidal individuals in slightly exposed areas. *S. purpuratus,* on the other hand, is mainly found intertidally in tide pools on very exposed, wave-beaten rocks. Natural and transplanted populations of *S. purpuratus*

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[1] This work was supported by grant GB-4038 from the National Science Foundation, and by NSF grant G-20901 to the Friday Harbor Laboratories for support of the junior author.

[2] This work was done during the tenure of an Established Investigatorship of the American Heart Association.

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were located in limited areas around Friday Harbor. In addition, several collections were made from the exposed coast, at Mukkaw Bay.

All animals were stored in running, oxygenated, sea water for at least one week prior to use. Water temperatures varied from 9.5° to 10.5° C., and the animals were maintained under relatively low light conditions (about 40 foot candles). Upon completion of experiments, animals were returned to similar tanks and observed over a period of several weeks for any signs of ill effects as a result of the experimental procedures.

METHODS

Partial pressures of oxygen in the external water (PiO_2) and in the coelomic fluid ($PcfO_2$) were measured using a Beckman Spinco gas analyzer (model 160). A special micro-cuvette permitted duplicate analyses of all samples (sample size: 0.05 ml.). The oxygen electrodes were calibrated with known gas mixtures or solutions equilibrated to known gas composition. Repeated sampling of coelomic fluid was accomplished by means of polyethylene catheters (P.E. 90) chronically implanted through a small incision in the peristomial membrane with the catheter tip protruding into the large coelomic cavity. Leakage around the catheter was prevented by a purse string suture in the peristomial membrane. Measurements of PO_2 were made at a temperature corresponding to the prevailing temperature in the animal's immediate surroundings.

Oxygen uptake (VO_2) was measured using a closed system at constant temperature (10.0° C.) (Lenfant, 1961). The partial pressure of oxygen in the respirometer was slowly reduced by the oxygen consumption of the animals. The size of the respiration chamber was adjusted until a reduction in PiO_2 from 150 mm. Hg to 10–20 mm. Hg occurred within a 6–8 hour period. VO_2 was calculated as microliters O_2/g. wet weight/hour. Both PiO_2 and $PcfO_2$ were measured in successive samples during the oxygen uptake experiments. An integral part of the investigation involved sampling of coelomic fluid from animals in their natural habitats. Such samples were obtained while SCUBA diving and collected into greased glass syringes by a needle inserted into the coelomic cavity through the peristomial membrane. The samples were quickly brought to the laboratory for analyses of $PcfO_2$.

RESULTS

Figure 1 shows a plot based on the average of three experiments utilizing six animals. Oxygen uptake (VO_2) (left ordinate, open circles) and PO_2 in coelomic fluid ($PcfO_2$) (right ordinate, filled circles) are plotted against oxygen tension in the ambient water PiO_2 in a closed system. In addition, values of $PcfO_2$ obtained from animals in their normal habitats have been plotted as a function of the PiO_2 at which the samples were taken (open squares, Fig. 1).

In well aerated water there was always a gradient in PO_2 between the coelomic fluid and the ambient water (Figs. 1 and 2). Upon lowering of the PiO_2, VO_2 stayed relatively unchanged down to values of 60–70 mm. Hg. During this decrease in the O_2 availability of the ambient water, the oxygen tension in the coelomic fluid characteristically showed two types of response patterns. In most cases $PcfO_2$ started to increase as soon as PiO_2 was lowered (Figs. 1 and 2). In other cases

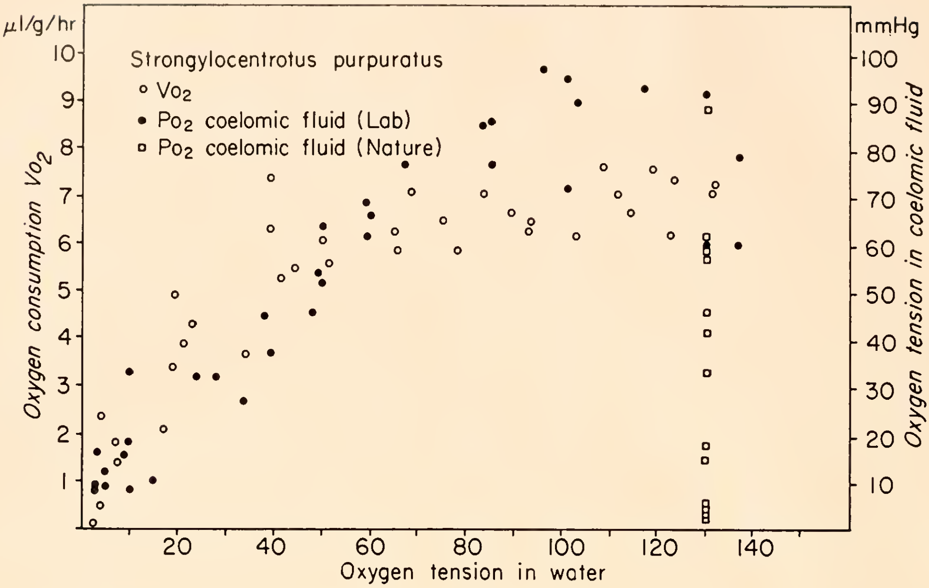

FIGURE 1. Oxygen consumption VO_2 (left ordinate, open circles), and partial pressure of oxygen in coelomic fluid, $PcfO_2$ (right ordinate, filled circles), of *Strongylocentrotus purpuratus* plotted against the partial pressure of oxygen in ambient water. $PcfO_2$ in samples obtained from animals in nature are also plotted (right ordinate, open squares).

it was maintained but in no case did $PcfO_2$ drop initially when PiO_2 decreased. The increased or maintained $PcfO_2$ rapidly reduced the gradient in O_2 tension between the coelomic fluid and the external environment. At the breaking point in oxygen uptake, at a PiO_2 of 60–70 mm. Hg, the internal and external media were essentially in equilibrium with respect to PO_2. Some of the values for $PcfO_2$ were considerably higher than the ambient water PO_2 (Figs. 1 and 2). Inadequate stirring in the coelomic fluid space may have been responsible for the seemingly paradoxical situation. However, Newell and Courtney (1965), working on respiratory movements in a holothurian, observed a similar response and maintained that the increased oxygen concentration of the coelomic fluid resulted from the animal's ability to absorb water and from a delayed transfer of oxygen from the respiratory trees to the coelomic fluid. Such an explanation, however, seems unlikely in the case of sea urchins.

When the PiO_2 dropped below values of 60–70 mm. Hg, both VO_2 and $PcfO_2$ dropped sharply and at about the same rate (Figs. 1 and 2). At very low oxygen tensions in the ambient water (19–20 mm. Hg) the VO_2 and $PcfO_2$ started to level off and were observed to remain largely unchanged at that low PiO_2 for periods of at least 5–6 hours. VO_2 was then reduced to approximately 15% of its value in air-saturated water. All animals subjected to experiments of this nature completely recovered after being transferred back to aerated sea water.

Figure 3 shows the composite results (average of 4 runs on each species) from experiments designed to study the gas exchange of sea urchins when placed in air.

The oxygen tension in the coelomic fluid followed a similar course for all three species. During the first hour after transfer to air, the $PcfO_2$ fell rapidly but subsequently levelled off and remained relatively unchanged from the second to at least the twelfth hour after initial exposure to air. All experimental animals recovered after being returned to normal sea water. The water and air temperatures during these experiments were 10° C. and 17° C., respectively.

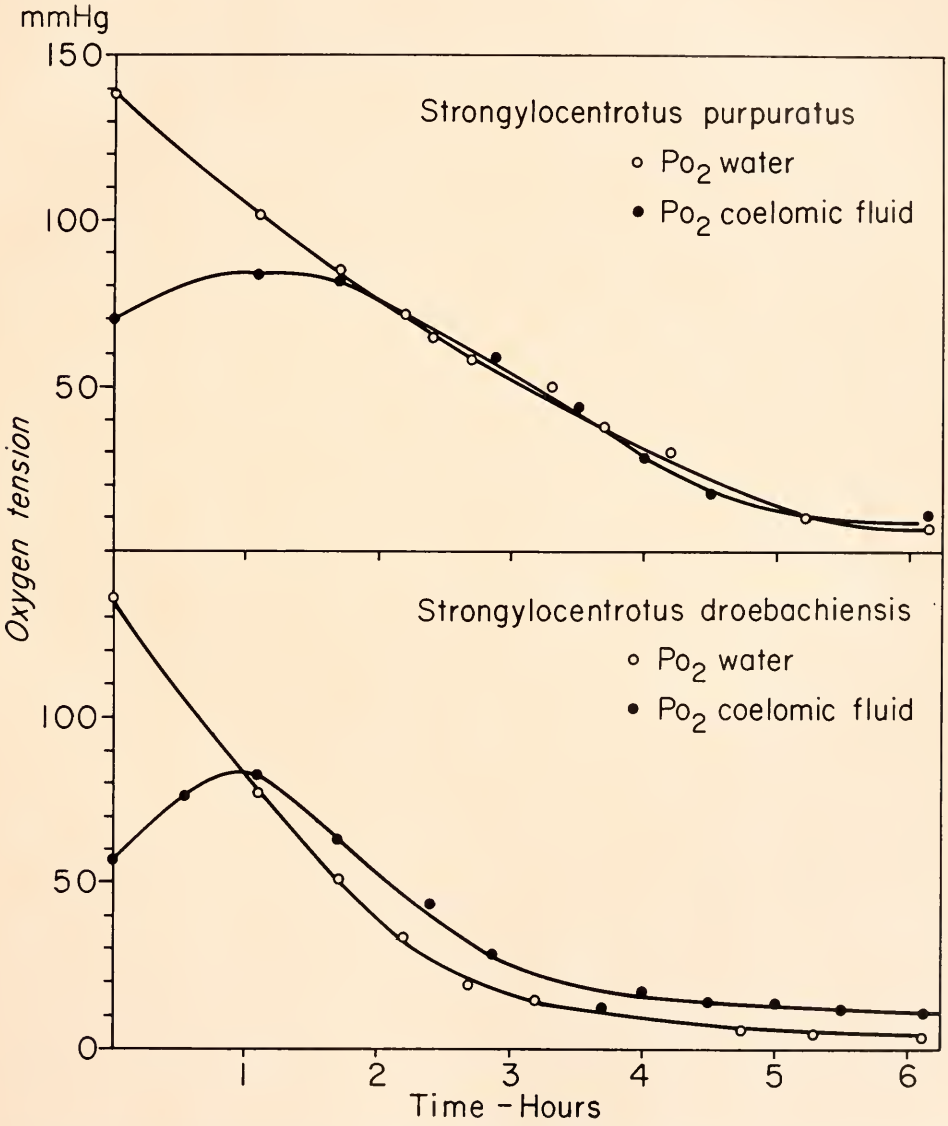

FIGURE 2. Partial pressures of oxygen in ambient water and coelomic fluid plotted against time for *S. purpuratus* and *S. droebachiensis,* when the urchins were in a closed respiration chamber.

Discussion

The water vascular system of echinoids constitutes their primary means for external gas exchange (Koller and Meyer, 1933, Steen, 1965). The system terminates externally in the podia or tube feet which make up the major surface for gas exchange. The external surface of echinoids is irrigated by ciliary currents. In addition, the movement of the tube feet increases the circulation of the external water. The water vascular system is lined internally by a flattened ciliated epithelium responsible for maintaining the water current carrying the respiratory gases. However, echinoids do not possess an effective system for internal oxygen transport.

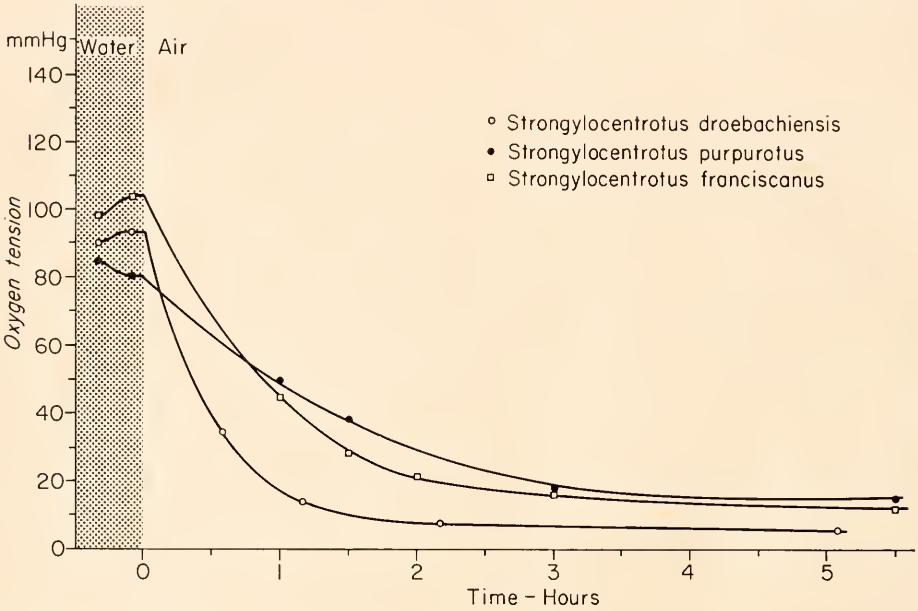

FIGURE 3. Partial pressure of coelomic fluid during air exposure of *S. droebachiensis*, *S. purpuratus* and *S. franciscanus*.

A large coelomic space lined with a ciliated epithelium constitutes the link between the water vascular system and the main mass of metabolizing tissue suspended within the spacious coelomic compartment. Although this arrangement does not insure an effective rate of transfer of oxygen from the ambient water to the respiring tissues, one must keep in mind the obvious role of the large coelomic fluid space as a storage compartment for the respiratory gases. Internal circulation based on fluid movement in discrete vessels and capillary beds does not offer a comparable storage factor. The importance of this in intertidal forms is amplified when considering the long interruptions of effective external gas exchange during tidal exposure. The role of the storage factor in respiratory exchange in holothurians has recently been alluded to by Newell and Courtney (1965).

The present data show that the oxygen tension of the coelomic fluid offers a good indication of the oxygen uptake of the animal, *i.e.*, that the rate of aerobic metabolism closely follows the oxygen availability in the internal environment bathing the metabolizing tissues. This in essence is carrying the concept of respiratory dependence a step further using the internal rather than the external environment as the reference for the oxygen uptake. Additional evidence for the close dependence of VO_2 on the PO_2 of the coelomic fluid was obtained in experiments involving exposure of the urchins to hyperoxygenated water (PiO_2: 350–500 mm. Hg). This procedure led to a rapid increase in $PcfO_2$ to values approaching that in the ambient water. Simultaneous monitoring of the oxygen uptake showed a conspicuous increase. It was imperative to extend these experiments over a long period of time (10–15 hours) in order to separate the true level of oxygen uptake from the mere storage of oxygen in the spacious coelomic fluid compartment.

Giese *et al.* (1966) suggest a similar dependence of VO_2 on the internal oxygen concentration, but their experiments were not designed to offer direct information on the problem.

The physiological significance of the close correlation between VO_2 and $PcfO_2$ becomes paramount in view of our data concerning the PO_2 levels in coelomic fluid sampled from animals in their normal environment. These data (represented by the squares in Figure 1) demonstrate a very large variation in $PcfO_2$ which in turn would indicate a similar variability in the actual oxygen uptake. One must now remember that all of these samples of coelomic fluid were obtained from animals located in water essentially air-saturated with oxygen ($PiO_2 > 130$ mm. Hg). Thus, in spite of a high O_2 availability in the ambient water the animal may decrease external respiratory efforts with a consequent reduction of coelomic fluid PO_2 and a reduction of overall O_2 uptake. In other words, rather than having its metabolic rate changed at the mercy of the external environment, the animal can make use of the phenomenon of respiratory dependence by lowering its internal O_2 level in well oxygenated water and thus reduce the oxygen uptake, thereby conserving energy when it is not needed.

The applicability of this concept, in a larger biological sense, seems rather important in many lower forms where only intermittent needs for a high operational ability are present, for instance, during feeding, escape and reproductive activities or in cycles depending on internal biochemical events.

The actual values for oxygen uptake of *S. purpuratus* obtained in the present study are much lower than those reported by Giese *et al.* (1966), but they compare well with the value listed for *S. lividus* (Spector, 1956).

The tolerance to air exposure as demonstrated in the present study is relevant to the problem of intertidal distribution of sea urchins. Our results show that during exposure to moist air at moderately increased temperatures (5° C. maximum increase) the external gas exchange can support a steady oxygen uptake at a level approximately 1/5 to 1/7 of the maximum in aerated water. Periods up to 15 hours of exposure to the described conditions were compatible with survival for the species investigated. The data warrant the suggestion that the endurance limit to air exposure at low tide may be more dependent on temperature increases and desiccation than on the ability of the urchins to exchange gases with air.

The capacity for compensatory alteration of the external gas exchange by means of the water vascular system became evident from the experiments involving gradual exposure to hypoxic water (Fig. 2). All animals responded to this by a compensation that maintained or even increased the $PcfO_2$ as the PiO_2 dropped. It is interesting to consider this compensation on the background of recent findings by Steen (1965) who showed that the oxygen uptake measured during the course of his experiments on *Strongylocentrotus droebachiensis* was only about one-tenth of the theoretical capacity of their podia. Steen relates this apparent inefficiency to limitations in the actual transport mechanisms of external and internal media. A compensation as demonstrated seems all the more likely when the limitation in external gas exchange is set by the movement of the respiratory media rather than by the thickness and area of the exchange surface.

The assistance of Stewart Grant and Dennis Willows in the diving operations of this study and the thoughtful comments by Dr. David Hanson are greatly appreciated.

SUMMARY

1. Oxygen uptake (VO_2) by sea urchins has been measured and correlated with partial pressures of oxygen in the ambient water (PiO_2) and in coelomic fluid ($PcfO_2$). $PcfO_2$ was also analyzed in samples obtained from animals in natural environments using SCUBA-diving technique. In addition, changes in $PcfO_2$ were recorded during prolonged air exposure of the animals.

2. The three species investigated, *Strongylocentrotus purpuratus*, *S. franciscanus* and *S. droebachiensis*, showed steady levels of VO_2 until PiO_2 had dropped to 60–70 mm. Hg. At lower PiO_2 the oxygen uptake decreased corresponding to the rate of decline of PiO_2.

3. Changes in $PcfO_2$ at decreasing PiO_2 closely paralleled the changes in VO_2, except for a common initial compensatory increase in $PcfO_2$. The data indicate that $PcfO_2$ represents a useful index of the level of VO_2.

4. $PcfO_2$ in samples obtained from urchins in their natural habitat showed large variations. Means by which the relationship between $PcfO_2$ and VO_2 may actively be used by the animals in their energy economy are discussed.

5. Air exposure of the urchins while monitoring changes in $PcfO_2$ suggests that external gas exchange in air is not a critical survival factor during tidal exposure.

LITERATURE CITED

Giese, A. C., A. Farmanfarmaian, S. Hilden and P. Doezema, 1966. Respiration during the reproductive cycle in the sea urchin, *Strongylocentrotus purpuratus*. *Biol. Bull.*, **130**: 192–201.

Handbook of Biological Data, 1956. Ed. W. S. Spector, W. B. Saunders Co., Philadelphia and London: 584 pp.

Koller, G., and H. Meyer, 1933. Versuche über die Atmung der Echinodermen (*Asterias rubens* und *Echinus miliaris*). *Biol. Zbl.*, **53**: 655–661.

Lenfant, C., 1961. A method for measuring VO_2 and VCO_2 of very small sea animals. *J. Appl. Physiol.*, **16**: 768–770.

Newell, R. C., and W. A. M. Courtney, 1965. Respiratory movements in *Holothuria forskali* (Delle Chiaje). *J. Exp. Biol.*, **42**: 45–57.

Steen, J. B., 1965. Comparative aspects of the respiratory gas exchange of sea urchins. *Acta Physiol Scand.*, **63**: 164–170.

SPERMATOCYSTS IN AEDES AEGYPTI (LINNAEUS)[1]

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It has been known for some time that groups of germinal cells in the testes of various mosquitoes are separated by delicate, membranous, transverse lamellae into a series of chambers or compartments (Hurst, 1890; Kulagin, 1907; Cholodkowsky, 1905; and Lomen, 1914) which can be referred to as *spermatocysts*. The aims of the present study were to determine the number and character of these compartments during the life span of the Bangkok strain of the Yellow Fever mosquito *Aedes* (*Stegomyia*) *aegypti* (Linnaeus) and to ascertain whether the spermatocysts would reflect the sexual activity of adults.

Larvae were reared in batches of 100 in 250 ml. of water in stender dishes and were well-fed. Pupae were sexed by examining the difference in their external genitalia (see Christophers, 1960) and allowed to emerge as adults in a cubic-foot screened cage, where they had continuous access to sugar water. The temperature varied from 25° to 30° C. The testes were dissected into a small drop of *Drosophila* saline (Ephrussi and Beadle, 1936) with fine needles and micro-forceps, and were examined with and without phase contrast microscopy, either without coverslipping or after slight flattening under a coverglass. Such flattening was often very useful when the testes were heavily encased in fat body, but in many cases even after flattening the fat body still completely obscured one or more portions of the testes so that accurate counts could not be made. The fat body could sometimes be removed by gently pushing the coverslip with a wet piece of filter paper which caused the testes to roll over in the wet whole mount. In a number of cases the fat body jacket was seen to be stripped away as the testes were being pulled out of the body. The number of compartments was generally counted and categorized at a magnification of 430 ×. Often two to four counts were made on the same region of a single testis and these were averaged. Frequently only one portion of a testis was sufficiently visible for accurate counts; hence, total numbers of cysts in Tables II through VI include only the complete counts. Wherever possible, the character of the cells in the different compartments was categorized as either undifferentiated (= spermatogonia, spermatocytes, very early spermatids) or as partially to fully differentiated. In some cases, however, only the number of cysts could be counted. In some other cases, the presence of maturation could be detected but accurate counts were not possible. Although it was often possible to discriminate partially- from fully-differentiated spermatocysts, this distinction could not always be made with certainty. Cells in the anteriormost portion of the testes were often very in-distinctly separated and the counting error is generally greater for this region than for the partially- to fully-differentiated cysts. The precise ages of pupae and adults were obtained by watching for the moment of ecdysis. These studies were made in the summers of 1960, 1961 and 1966.

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[1] This research was sponsored by N. I. H. Grant GM 06021 and by N. I. H. Development Award GM 21,529. Scientific Article Number A1289, contribution number 3829 of the Maryland Agricultural Experiment Station.

I am indebted to Dr. Arden O. Lea, Elliot S. Krafsur and Elizabeth Jones for many helpful comments on the manuscript.

OBSERVATIONS

Larvae were dissected daily from the first through the seventh days after hatching, independently of their particular stadium. Forty-two gonads dissected from 34 larvae were identified as testes. The number of spermatocysts ranged from three in one-day-old larvae to a maximum of 24 in a 6-day-old fourth stage larva. The mean numbers of cysts for the larval period varied from 3.8 to 22.5 (Table I). Only one out of 8 testes from five-day-old larvae showed evidence of early differentiation of the germ cells in the posteriormost compartment. The cells in this case were pyriform spermatids. In none of the larvae examined were fully differentiated spermatozoa observed. Last stage larvae had from 15 to 24 testicular compartments, with an average of about 20. During larval life, the testes increased 8 to 9 times in length and changed from small ovoid organs into long pyriform structures. Most of the testes dissected from larvae were free of fat body up to the fourth stage. In fourth stage larvae, one of the testes tended to be noticeably larger than the other in the same individual. It is estimated that the number of spermatocysts increases about five times during larval life. The maximum number of cysts is reached several days before pupation.

TABLE I

Daily spermatocyst counts of Aedes aegypti (L.) during the larval stage

Days after hatching	No. larvae dissected	No. testes	No. counts	Number of spermatocysts	
				Range	Mean
1	4	4	6	3–5	3.8
2	5	6	10	4–10	7.3
3	5	7	7	10–18	14.3
4	6	7	15	16–22	19.9
5	7	8	11	15–21	18.0
6	4	4	2	21–24	22.5
7*	5	8	13	9–23	16.4

* Data collected 1966; other data 1960–1961.

During the pupal stage the number of spermatocysts ranged from 9 to 29, with means fluctuating around 19 (Table II), thus indicating that the number of testicular compartments remains essentially the same as that of fourth stage larvae. Although 8 out of 9 testes dissected from 6 newly emerged "white" pupae (0–8 minutes old) had from one to four cysts (Table II) with spermatids in the spindle form or with flagellum formation beginning, some other batches of newly emerged pupae (0–10 minutes old) which were examined during these studies had no differentiating cysts at all (11 testes from 6 pupae; data not shown). Indeed, a few testes had not begun to differentiate sperm for as long as 21 hours after pupation.

In some batches of pupae, a few spermatocysts were matured as early as five hours; in others, fully matured spermatozoa were not seen until near the end of the first day. The number of differentiated cysts definitely increases in old "black" pupae about to emerge as adults (that is, within pharate adults), when as many as 13 matured cysts were found in one case. The matured sperm were not active within these cysts. Sperm were never observed in the sperm ducts during the pupal stage.

Spermateleosis (process of differentiation of spermatozoa from spermatids) always begins within the posteriormost compartment of the testes and proceeds anteriorly until as much as 75% to 80% of the gonads may have differentiated cysts.

TABLE II

Spermatocyst counts of Aedes aegypti (L.) during the pupal stage

Age	No. dissected	No. testes	Number of cysts/testis							
			Extent of differentiation						Total†	
			None		Begun		Complete			
			Range	Mean	Range	Mean	Range	Mean	Range	Mean
0–8 min.*	6	9	11–17	14.8	0–4	2.3	0	—	13–21	17.1
25–45 min.*	5	9	14–19	16.7	1–2	1.2	0	—	15–20	17.5
3½–4 hr.	5	6	15–29	20.0	0–4	0.6	0	—	15–29	20.6
5 hr.	6	12	9–22	15.3	0–		0–5	1.8	9–23	17.0
5–6 hr.	5	8	15–24	21.3	0–		0–		15–24	21.3
16½–17½ hr.	9	14	11–23	17.7	0.4	1.5	0–		13–23	19.2
18–21 hr.	10	13	14–23	18.9	1–4//	2.0	0–		15–25	19.9
21–22 hr.*	4	7	10–19	14.8	0–1	—	3–6	4.7	15–23	20.1
24 hr.	3	5	13–20	16.0	1–4#	2.2	0–8¢	3.4	18–24	21.6
27–29 hr.	3	4	11–15	13.0	—	—	4–6¢	5.0	16–20	18.0
Old pupaeφ	7	9	9–12	10.8	0–1	0.2	6–12¢	9.3	18–24	20.6
Old pupaeφ*	5	8	6–10	7.9	—	—	6–13	8.7	12–20	16.5

† Totals do not include partial data.

* 1966 data; all other data are for 1960–1961.

φ Pharate adults.

// Based on 10 measurements.

Based on 3 measurements.

¢ Combined partially to fully differentiated cysts.

The anteriormost region of the testis always maintains an undifferentiated zone of cysts. Although there is a tendency for a number of cysts in both testes within an individual to begin to mature at the same time, individuals have been seen where maturation had begun in one testis and not in the other. There was no correlation between the presence or absence of a lumen in the vasa efferentia and the maturation of the testes.

Within the matured testis the non-differentiated anterior portion is made up of spermatocysts in bands or layers one to three or more cells thick in optical section. The cells are relatively large and spherical and each has a large nucleus. (Before the testis matures, all of the germ cells have this appearance and the cysts are small anteriorly and progressively tend to enlarge posteriorly.) The spermatids pass through 8 stages to become mature spermatozoa (Krafsur, 1964). After passing through the pyriform and spindle stages, the cells progressively elongate and a flagellum is formed. Cysts with early differentiating spermatids do not have a distinct color. As differentiation proceeds, the cysts take on a distinct yellowish brown cast with transmitted light. Differentiating cysts tend to be larger than non-differentiated ones. The terminal cyst is the largest testicular compartment; and, after the first day of adult life, the spermatozoa actively move about within it, often

TABLE III

Spermatocyst counts on testes of unmated Aedes aegypti (L.) during the adult stage

Age and status	No. dissected	No. testes	Numbers of cysts/testis						
			Extent of differentiation						
			None		Partially to fully differentiated			Total†	
			Range	Mean	Range	Mean	%	Range	Mean
Newly emerged; unrotated	15	18	10–17	12.7	5–18	9.8	43.1	18–34	22.5
0 hour	7	12	5–11	9.2	3–14	9.5	48.2	13–25	19.7
5 hour*	6	11	5–9	7.5	7–12	9.8	56.6	15–21	17.3
¼ rotated*	5	7	7–16	10.5	8–11	9.5	48.8	16–25	20.0
10 hour (½ r)*	4	8	6–11	8.1	7–13	10.0	53.8	15–22	18.6
½ rotated	9	13	6–17	11.2	3–14	10.5	47.0	14–31	21.7
15 hour*	6	12	6–12	9.1	5–13	8.4	46.9	12–24	17.9
0–1 day	4	7	5–11	7.2	7–10	8.2	53.7	12–18	15.4
16½-24 hour; rotated	9	14	9–15	11.2	6–14	9.3	45.5	18–26	20.5
1 day	10	13	6–10	9.1	4–9	6.7	43.3	11–19	15.8
24 hour*	5	8	6–16	9.5	6–11	7.7	44.0	12–24	17.5
2 days*	11	17	4–12	6.6	3–9	5.6	45.7	7–18	11.9
3 days*	7	12	3–10	7.0	3–6	4.8	41.9	7–16	11.9
4 days*	6	11	4–9	5.5	3–6	4.4	43.6	7–15	9.9
5 days*	5	10	4–13	7.4	2–8	4.5	37.7	7–18	12.1
6 days*	5	9	5–10	6.4	2–5	3.7	34.5	7–14	10.0
7 days*	6	10	5–14	7.5	2–6	3.9	35.8	8–18	11.2
7 days	10	18	3–11	8.2	2–5	3.0	27.1	7–14	11.2
10 days*	7	13	5–10	8.0	2–6	3.4	29.8	9–16	11.4
2 weeks	6	10	4–9	6.5	2–6	3.9	35.1	8–12	10.4
2 weeks	10	20	5–16	7.4	2–6	3.8	35.4	8–13	11.2
3 weeks	10	18	3–18	5.4	2–5	2.6	32.4	5–12	8.0
4 weeks	10	18	3–9	7.0	0–5	2.7	30.3	5–12	9.7
5 weeks	5	8	3–8	4.9	2–5	2.9	36.6	5–11	7.8
6 weeks	5	8	2–8	5.3	1–5	2.2	31.5	5–13	7.5

† Totals do not include partial data.

* Data collected 1966; all other data collected 1960–1961.

in dense, violently spinning whorls. The long threadlike cells in fully differentiated compartments are tightly wound into ovoid balls, when the sperm are inactive *in situ*. The testes of pupae and especially of the adults exhibit a wide number of variations of the pyriform shape. The anterior end generally tends to be recurved and the posterior end is either cuplike or in the shape of a funnel. The testis may be bent into a C-shape. The middle portion may be compressed like a waist.

Testes were removed from unmated adults from the time of their emergence through the sixth week. The number of spermatocysts did not increase significantly at the time of adult emergence or thereafter. In the adult, the spermatocyst lamellae frequently did not prevent active sperm from being able to move from chamber to chamber in the differentiated region, but the sperm never moved into the non-differentiated zone. The number of cysts was found to range from five to 34, with means of 7.5 to 22.5 for the period of study (Table III). Undifferentiated compartments ranged from two to 18 (means of 5.3 to 12.7) and partially to fully matured cysts varied from none [2] to 18 (means of 2.2 to 10.5) (Table III).

Out of 50 individual comparisons, 8% of the adults examined had both testes of essentially the same size, while the remainder had one testis distinctly smaller (by a factor of 1.3-fold) than the other in the same individual. Both large and small testes tended to have approximately the same number and character of spermatocysts. Over the 6-week period of study, no significant change could be detected in the length of the testes in the 44 cases available for comparison. The length of the large testes ranged from 340 to 737 microns, and the small testes varied from 150 to 660 microns. There was no correlation between the size of a testis and the direction of rotation of the terminalia among 10 individuals studied in this regard.

During the first 24 hours of adult life, the male's terminalium rotates 180° and the posteriormost compartment of each testis opens and a certain number of sperm descend the spermatic duct (vas efferens plus deferens) and begin to fill the seminal vesicles. Among many recently emerged adults, sperm were not present in the vas efferens of one testis but were present in the duct of the other and subsequently generally one duct contained more sperm than the other in the same individual. In 12 individual comparisons, there was no correlation between the presence or number of sperm in the ducts and the size of the testes. While one testis clearly may be the first to provide a portion of the initial supply of sperm to the seminal vesicles, sperm from both testes are required to fill the vesicles.

During the first 24 hours of adult life, the mean number of non-differentiated cysts per testis ranged from 7.2 to 12.7 (with an overall mean of 9.6) and the differentiated cysts from 6.7 to 10.5 (with an overall mean of 9) (Table III). Although sperm begin to fill the postgonadal system during this period, no significant differences in the number or character of the cysts could be detected. With the present material a deletion of two mature cysts per testis could *not* have been detected.

Between the second and tenth days of adult life, when the sperm have already filled the postgonadal system, the mean number of undifferentiated cysts per testis ranged from 5.5 to 8.2 (with an overall mean of 7.1) and the matured cysts from 3 to 5.6 (with an overall mean of 4.2). These overall means differ from those of

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[2] The duct from this testis possessed numerous spermatozoa throughout its length.

0- to one-day-old adults by 2.5 fewer undifferentiated cysts and by 4.8 fewer differentiated cysts per testis.

Jones and Wheeler (1965) reported 700 sperm in mature cysts, 740 sperm in the spermatic ducts, and from 3700 to 6309 sperm in the seminal vesicles of unmated *Aedes aegypti.* The filled postgonadal system would thus have from 4440 to 7049 spermatozoa. If these values are correct, spermatozoa within 6.3 to 10 matured cysts would be needed to fill the postgonadal system. The mean deletion of 4.8 matured cysts per testis thus fits in with this requirement, and could account for a supply of 6720 spermatozoa within the postgonadal system of *A. aegypti.* At least 2000 sperm could reach the postgonadal system during the first 24 hours after emergence, and the remainder be delivered shortly thereafter.

TABLE IV

Spermatocyst counts on testes of Aedes aegypti (L.) kept with approximately equal numbers of females for four to 7 weeks

Age	No. dissected	No. testes	Numbers of cysts/testis						
			Extent of differentiation					Total†	
			None		Partially to fully differentiated				
			Range	Mean	Range	Mean	%	Range	Mean
4 wks.	6	8	5–12	7.6	1–3	2.3	24.9	7–15	9.9
6 wks.	4	8	2–9	6.0	1–4	2.3	29.7	5–12	8.6
7 wks.	5	9	2–8	5.5	1–5	2.4	33.0	4–12	7.4

† Totals do not include partial data.

During the six-week period of study, the total number of spermatocysts in unmated males gradually declined from 22.5 to 7.5 (by a factor of 3); the undifferentiated cysts decreased from 12.7 to 4.9 (by a factor of 2.6); and the differentiated cysts were reduced from 10.5 to 2.2 (by a factor of 4.8) (Table III). The mean percentage of compartments with differentiated sperm decreased from 56.6% to a minimum of 27.1%, with an overall mean of 41.1% differentiated cysts for the entire 6-week period of study. Viewed as a whole, there is a distinct and significant trend for both undifferentiated and differentiated cysts to decline with age in unmated adults, that is, in the absence of any loss of sperm from the reproductive system.

If each mature cyst produces 700 spermatozoa, then the following calculations can be made from the data in Table III: (1) About 13,500 sperm are present in both testes before any or very few of them descend to the postgonadal system in the newly emerged adult. (2) After 24 hours, 2720 to 4120 sperm have left the testes. (3) During the next 9 days, there are from 4200 to 7840 sperm in the testes and from 5660 to 9300 in the postgonadal system. (4) If the number of sperm in each mature cyst does not change with time, then the amount of sperm in the testes should gradually decrease as the supply in the postgonadal system increases in unmated males. Between the second and tenth days, 3640 sperm should leave the testes, and, between the second and sixth weeks, 2380 sperm should leave the testes.

It is possible, however, that once the postgonadal system is filled, relatively few or no additional sperm would be added thereafter. If this were the case, spermatocyst walls could break down, leaving the same numbers of sperm within the differentiated region of the testes but with fewer spermatocysts detectable therein. With the present data, it is not possible to decide which of these is the case.

As shown in Table IV, data from males which had been caged continuously with approximately equal numbers of females for four, 6 and 7 weeks did not differ in number or character of spermatocysts from the data obtained from unmated males of the same ages (Table III).

TABLE V

The number and character of spermatocysts in Aedes aegypti (L.) after multiple copulation

Sex ratio and cohabitation time	Number males dissected and age when dissected	No. testes	Numbers of cysts/testis						
			Extent of differentiation					Total†	
			None		Partial-complete				
			Range	Mean	Range	Mean	%	Range	Mean
1:10/3 days	10/8 days	16	6–14	8.9	2–4	3.0	26.0	9–17	11.9
controls	6/8 days	11	5–14	8.8	1–7	3.7	30.2	6–20	12.2
1:20/1 day	11/3–4 days	17	3–13	8.7	2–10	4.8	36.9	10–17	13.6
controls	9/3–5 days	16	4–17	9.3	2–7	4.2	31.6	6–18	13.3
1:20/2 days	3/7 days	6	7–10	9.0	2–7	5.5	38.0	12–17	14.8
controls	6/7 days	10	6–13	8.8	2–5	3.1	26.1	9–16	11.9
1:20/4 days	3/14 days	6	6–11	8.2	2–7	3.8	31.4	10–14	12.3
controls	6/14 days	12	4–17	8.1	2–6	3.3	30.5	8–21	11.4

† Totals do not include partial data.

Since it had been found that the reproductive systems of males kept in the presence of equal numbers of females for as long as 7 weeks could not be distinguished from those of unmated controls (Table IV), a series of cages were set up containing varying combinations of previously unmated adults: (1) one cage of 10 males with 100 previously unmated females, (2) four cages of one male with 20 females and two cages of five males with 100 females. The adults were allowed to co-habit for one to four days and the males were dissected. Frequent matings were observed but the sexual history of individual males was not determined.

As shown in Table V, when the sex ratio was 1:10 or 1:20, the number and character of the spermatocysts were basically the same as those of the unmated controls. However, in 60% of the males the spermatic ducts and seminal vesicles contained very few spermatozoa and these individuals generally had noticeably reduced accessory gland secretion, particularly when the sex ratio was 1:20. Presumably, those males with a reduced supply of sperm and accessory gland material mated with more females than those whose supplies were not strikingly reduced. Thus, depletion of sperm from the postgonadal system and of accessory gland material did not appear to affect the general character of the testes.

This finding fits with the data of Jones and Wheeler (1965) which showed that, after males had been force-mated repeatedly, the posterior chamber of their testes still had many spermatozoa (mean of 741). Together these data show that after repeated matings only the sperm in the postgonadal system are used up and that testicular sperm are not drawn down to replenish the supply as it is being removed.

To explore this problem further, three cages were set up, each with five males to 30 females; and, after co-habiting for 24 hours, the males were isolated for one, two or three days before being dissected. After one day, three out of five males had shrunken seminal vesicles with very few spermatozoa, and the ducts leading to them had very few if any sperm; the other two males had an obviously reduced supply of vesicle sperm. Two days after isolation, three out of five males had shrunken vesicles with very few spermatozoa, but two males had replenished the sperm within the seminal vesicles. After three days, four out of five males had replenished the sperm in their vesicles, and their accessory glands were filled with secretion. Only one male still had very few sperm within his vesicles. Essentially the same results were obtained with 5 males to 100 females (5 cages; co-habitation time one, two, and four days).

TABLE VI

The number and character of spermatocysts in Aedes aegypti (L.) after being isolated following multiple copulation

Days isolated and sex ratio	No. testes	Numbers of cysts/testis						
		Extent of differentiation					Total†	
		None		Partial-complete				
		Range	Mean	Range	Mean	%	Range	Mean
1 day 1:6	3	7–11	8.7	1–4	2.3	21.6	10–12	11.0
2 1:6	9	6–22	12.2	1–4	2.4	22.5	8–23	14.4
3 1:6	6	5–9	6.8	3–5	3.7	36.3	9–13	10.7
2 1:20	8	7–11	8.7	2–6	3.5	28.5	9–15	12.3
controls	7	4–17	9.1	1–6	3.5	26.8	6–18	12.2
5 1:20	13	5–11	7.4	2–6	3.9	35.7	8–16	11.2
controls	13	5–10	8.0	2–6	3.4	29.8	8–16	11.4

† Totals do not include partial data.

As shown in Table VI, when the sex ratio was 1:6, there does not appear to be much difference between the number of differentiated cysts in testes of depleted males and those of males which have largely replenished their sperm supply. If 4440 to 7049 sperm were removed from the testes to replenish their postgonadal supply, then 6 to 10 matured cysts should have been needed and this would require more sperm than would have been present within the 4.8 matured cysts which were available in both testes. Three days after being isolated from females, however, the males, after mostly replenishing their sperm supply, had *more* mature cysts than the depleted males. Since there was an *increase* of 3.5 undifferentiated cysts per testis two days after males were isolated from females, and since there was a *decrease* of 5.4 undifferentiated cysts after the sperm supply had been replenished on the third day, it seems likely that the 4.8 matured cysts were indeed all used up and then replaced by maturation of new cysts derived from the undifferentiated zone of the testes. Thus, it can be calculated that the 4.8 already matured cysts from both testes would contribute only 3360 sperm to the postgonadal system, and that the 5.4 extra undifferentiated cysts in each testis would produce a total of 10.8 matured cysts for both testes: of these 7.4 would replace and thus account for the 3.7 matured cysts seen in each testis of the replenished male, and the other 3.4 cysts would contribute 2380 sperm to the postgonadal system to bring the total supply there to 5740.

Discussion

During the larval life of *Aedes aegypti,* the testes grow in size and the germ cells greatly increase in numbers within them apparently near or around the time of each larval ecdysis. In some old larvae (pharate pupae) spermatids may just begin to differentiate in the posteriormost compartment of the testes. The general shape of the gonads does not depend upon the presence of germ cells, as evidenced by those males with agametic testes (Jones, 1961). Although agametic testes may still possess a number of compartments, mostly or entirely at the anterior end, they generally possess far fewer than do normal testes. Agametic testes are always smaller than normal, thus showing that the growing number of germ cells leads to a general increase in size of the testes (Jones, 1961).

The present observations indicate that differentiation of spermatids may begin shortly before pupation occurs (that is, in pharate pupae) or they may not begin for 6 to 21 hours. It is of considerable interest that differentiation can begin in one testis without necessarily simultaneously beginning in the other. The entire process of differentiation of spermatids into spermatozoa within a single cyst may be completed within a five-hour period. Maturation always begins in the posteriormost cyst. As many as 6 cysts may be maturing at the same time within a testis. Matured cysts were found in all pupae after the first 24 hours. Maturation of spermatocysts is preeminently a pupal event.

In old pupae just about to emerge as adults (that is, in pharate adults) 8.7 to 9.3 matured cysts were present in each testis. It can be calculated from this that there are 6090 to 6510 spermatozoa within each testis at this time.

The present calculations indicate that while sperm begin to fill the postgonadal system shortly after the adults emerge, this process is not completed until the second day of adult life. It is estimated that about 10 matured cysts are required to fill the postgonadal system with about 7000 spermatozoa and that both testes must contribute to this supply.

In unmated males the number of spermatocysts declines in the absence of any loss of sperm from the reproductive system. It is not clear whether this involves an increase in the numbers of sperm within the postgonadal system as their numbers decrease in the testes or whether there are no changes in the numbers of spermatozoa within different portions of the reproductive system but only a breakdown of spermatocyst lamellae.

After inseminating 6 females, the male quickly uses up all or nearly all of the sperm in his postgonadal system but the numbers of sperm in the testes are not immediately affected or drawn upon. When such males are isolated from females, they replenish the sperm in their reproductive system in two to three days and the numbers of spermatocysts do not clearly reflect this change. It is suggested that replenishment cannot be achieved solely by the use of all the sperm within the already matured cysts of the testes but requires the formation and maturation of about 11 extra cysts.

SUMMARY

1. During the larval life of *Aedes aegypti* (L.), the testes greatly increase in size and numbers of germinal cells, and the number of compartments (or spermatocysts) increases about five times, to a maximum of 24, usually several days before pupation. Although the germ cells may begin the process of differentiation of spermatids into spermatozoa within the terminal cysts of the testes just before pupation occurs, fully differentiated spermatozoa were never observed in larvae. Generally one testis is smaller than the other in fourth stage larvae, and this difference tends to persist throughout life.

2. Although a significant increase in the number of spermatocysts could not be detected during pupal life, as many as 29 cysts were found among the testes examined during this period. Spermatids may transform into fully differentiated spermatozoa within five hours. While the beginning of differentiation of the spermatids may be delayed for as long as 21 hours after pupation, differentiated sperm were always found after the first 24 hours. The number of differentiated cysts increases during pupal life and it is calculated that 12,000 to 13,000 spermatozoa are formed by both testes. Spermatozoa were never observed in the spermatic ducts during the pupal stage.

3. Although a significant increase in the number of spermatocysts could not be found during adult life, a maximum of 34 cysts were found among newly emerged adults. The number of spermatocysts definitely declines with the age of unmated adults. Spermatozoa begin to fill the postgonadal system during the day of adult emergence. It is estimated that complete filling requires two days, and that the sperm in about 10 cysts are required.

4. When the male uses up most or all of the sperm within his postgonadal system after multiple matings, two to three days are required to replenish the sperm supply. It is suggested that this must involve the formation and maturation of about 11 extra cysts, most of which are needed for replacement within the testes, the others contributing to the supply of postgonadal sperm.

LITERATURE CITED

Cholodkowsky, N., 1905. Über den Bau des Dipterenhodens. *Zeitschr. wissenschaft. Zool.*, **82**: 389–410.

Christophers, S. R., 1960. *Aedes aegypti.* The Yellow Fever Mosquito. Its Life History, Bionomics and Structure. Cambridge Univ. Press., 737 pp.

EPHRUSSI, B., AND G. W. BEADLE, 1936. A technique for transplantation for *Drosophila*. *Amer. Nat.*, **70**: 218–225.

HURST, C. H., 1890. The post-embryonic development of a gnat (*Culex*). *Trans. Liverpool Biol. Sci.*, **4**: 170–191.

JONES, J. C., 1961. The internal reproductive anatomy of sterile male *Aedes aegypti* (Linnaeus). *Mosq. News*, **31**: 118–119.

JONES, J. C., AND R. E. WHEELER, 1965. Studies on spermathecal filling in *Aedes aegypti* (Linnaeus). I. Description. *Biol. Bull.*, **129**: 134–150.

KRAFSUR, E. S., 1964. The spermatogenesis of *Aedes aegypti* (L.). M.S. thesis, Univ. of Md., College Park, Md., 73 pp.

KULAGIN, N., 1907. Zur Naturgeschichte der Mücken. *Zool. Anz.*, **31**: 865–881.

LOMEN, F., 1914. Der Hoden von *Culex pipiens* L. (Spermatogenese, Hodenwandungen und Degenerationen). *Jenaische Zeitschr. Naturwissen.*, **52**: 562–628.

NITROGENOUS EXCRETION IN THE TROPICAL SEA URCHIN DIADEMA ANTILLARUM PHILIPPI

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Echinoids are considered to be predominantly ammonotelic in the excretion of their nitrogenous metabolic end products (Prosser and Brown, 1961 ; Nicol, 1960). Other excretory products have, however, been found in the perivisceral fluid and tissue of echinoids or in the surrounding sea water of vessels in which specimens have been enclosed. Delaunay (1931) found that considerable amounts of amino acids were excreted by the urchins *Paracentrotus* and *Strongylocentrotus,* as well as small amounts of urea, uric acid and other purines. Conheim (1901) found urea, amino nitrogen, ammonia and purine bases in the coelomic fluid of urchins. Sanzo (1907) found urea in several species and Myers (1920) found creatine, creatinine, uric acid, urea and ammonia in *Strongylocentrotus franciscanus.* Van der Heyde (1923) found only uric acid in the coelomic fluid and intestine of *Arbacia,* while Przylecki (1926) found only traces of uric acid in echinoids. Boolootian (1961) has listed the amounts of nitrogenous excretory elements in the perivisceral fluid found in a number of echinoids.

Excretion studies on echinoids have been mainly concerned with temperate and cold-water species. The determination of the excretory products and rate of excretion of *Diadema antillarum* is thus of interest in terms of the physiology of a sea urchin of widespread occurrence in the tropics.

The study was supported by a grant in aid of research from the National Research Council of Canada.

METHODS

Experiments were performed on freshly collected specimens of 5 to 7 cm. test diameter. Specimens were placed in covered glass jars filled with a measured amount of filtered sea water, of between 2 and 4 liters. A control jar contained no urchin. Tests were run for 4 hours at approximately sea temperature (26–28° C.). At the completion of the trial the urchins were removed and the water filtered to remove faecal matter and other solids. Analyses for excretory products in the water were begun immediately.

Descending paper chromatograph techniques were used as qualitative tests for excretory products in the "excretory water" as well as in the pericisceral fluid. After electrolytic desalting (Baird & Tatlock desalting apparatus) 0.5 ml. of fluid or water was evaporated on a paper pad. This pad was then affixed with a plastic clip to the top of the paper chromatogram strip. Appropriate amounts (1–10 μg., depending on sensitivity of method) of reagent quality samples of the substances being tested for were applied to paper pads and affixed to adjacent paper strips as standards, according to the methods of Smith (1960).

Urea was tested for by the methods of Block *et al.* (1958), using phenol with sodium hypochlorite and with acetone and dimethylaminobenzaldehyde (Smith, 1960). Uric acid and other purines were tested for by the method of Block *et al.* (1958), using diphenylcarbazone on acidified mercuric acetate in ethanol and by ultra-violet light (Smith, 1960). Creatine and creatinine were tested for by the picric acid method (Block *et al.*, 1958) and amino acids were identified with ninhydrin.

Ammonia and total nonprotein nitrogen were determined quantitively by microdiffusion methods (Conway, 1962). Kjeldahl treatments of sea water samples prior to diffusion determinations were modified to use 200 ml. of water according to the method of Barnes (1959). Amino acid quantitative determinations were a microdiffusion modification of the ninhydrin method of Sobel *et al.* (1945). Tests for urea by diffusion were also run according to Conway (1962), using urease tablets (British Drug Houses). Filtered sea water with and without urease were run as controls and replicate samples were tested.

Table I

Mean hourly production in μg. of excreted nitrogen by Diadema

Total N.P.N.	NH_3N	$\%NH_3N$	Amino N.	% Amino N.	Number of animals	Duration exp. in hours
162	99	61	42	26	12	4
245	157	64	71	29	12	4

Results

In spite of repeated attempts to distinguish them in both perivisceral fluid and in the "excretory water" of *Diadema*, urea, uric acid or other purines, creatine and creatinine were not found by the methods used. Since these methods were sensitive to a few micrograms of the detectable substances, it would appear that urea, purines and creatine are only present in very minute amounts if indeed they are nitrogenous excretory products. The amounts of urea, purines, and creatine and creatinine noted by Boolootian (1961) for other echinoids were of the same order as the amounts detectable by the chromatographic methods used here.

Amino acids, however, were found in sensible amounts, together with ammonia. The results of analyses of excreted ammonia nitrogen, nonprotein nitrogen and amino acid nitrogen in excretory water are shown in Table I.

The highest proportion of excreted nitrogen occurred as ammonia nitrogen in two series of experiments. In the first series, urchins were freshly collected, while in the second series the specimens had been previously fed for 12 hours on a diet of fish meal.

The results of analyses of samples of perivisceral fluid showed no urea, purines, creatine or creatinine. Substantial amounts of amino acids were detected on paper chromatograms, however, and ammonia nitrogen was also present. The amounts of ammonia nitrogen in the fluid were found to vary between 42 and 148 μg. per

100 ml. of fluid in freshly collected specimens. The mean content of 20 specimens was 100 μg. of ammonia nitrogen per 100 ml. of fluid. The ammonia nitrogen content of the fluid of animals which had been fed on a protein diet for 24 hours was markedly higher. Amounts of ammonia nitrogen in 16 specimens varied between 255 and 645 with a mean of 374 μg. of nitrogen per 100 ml. of fluid.

Since no specific excretory organ is known for echinoids it is of interest to compare the amounts of ammonia nitrogen found in the various tissues. The amounts of ammonia nitrogen were obtained by grinding a known weight of tissue in distilled water which was free of ammonia and subsequently determining the ammonia by diffusion (Conway, 1962).

TABLE II

Mean ammonia nitrogen concentrations in μg./100 gm. in various tissues of Diadema

Organ	Ammonia N. μg./100 gm.	No. specimens analyzed
Oesophagus	159	12
Caecum	195	12
Foregut	317	12
Hindgut	400	12
Rectum	155	12
Gonad	68	12
Muscle	61	12
Gills	75	12

The results of determinations of tissue ammonia contents are shown in Table II. The results show increasing concentrations of ammonia nitrogen in the gut towards the rectum. Concentrations rose from 159 μg. in the oesophagus to 400 μg. per gm. of tissue in the hind gut. Concentrations in the gills, gonads and muscle were comparatively low.

DISCUSSION

Like most other sea urchins, *Diadema* excretes the largest proportion of its nitrogenous waste as ammonia but substantial amounts of amino acids were also excreted. The amounts of ammonia nitrogen found in the perivisceral fluid are comparable to those found in other urchins. Delaunay (1931) recorded a value of 240 μg./100 ml. in *Paracentrotus lividus* and Myers (1920) found 80 μg./100 ml. in *Strongylocentrotus franciscanus.* Unlike other urchins whose excretory physiology has been investigated, no urea, purines, creatine or creatinine have been found.

The ammonia content of the various tissues is of interest for it suggests increased excretory activity towards the distal end of the hind gut. Sections of the hind gut just preceding the rectum had more than twice the amount of tissue ammonia than in the oesophagus and caecum. Progressive increase in tissue ammonia along the gut in insects has been interpreted as denoting areas of excretory function (Lennox, 1940; Staddon, 1955). However, the degree of differences in ammonia content found in insects was far greater than occurred here in *Diadema.* The hind gut was considered to have an excretory function in echinoids by Van der Heyde (1923) and Delaunay (1931).

SUMMARY

Diadema antillarum is ammonotelic in its excretion of nitrogenous waste products. It excretes approximately 60% of its total nonprotein nitrogen as ammonia and approximately 30% as amino acids. No urea, uric acid or other purine bases were found to be excreted. A progressive increase in tissue ammonia content in the intestine towards the rectum suggests that the hind gut has an excretory function.

LITERATURE CITED

BARNES, H., 1959. Apparatus and Methods of Oceanography. Part One. Chemical. George Allan and Unwin Lt., London.

BLOCK, R. J., E. L. DURRUM AND G. ZWEIG, 1958. A Manual of Paper Chromatography and Paper Electrophoresis. Second Edition. Academic Press Inc. New York.

BOOLOOTIAN, R. A., 1961. Physical properties and chemical composition of perivisceral fluid: Echinodermata. Reprinted from Biol. Handbook: Blood and other Body Fluids. Federation of American Societies for Experimental Biology. pp. 339–344.

COHNHEIM, O., 1901. Versuche über Resorption, Verdauung und Stoffwechseln von Echinodermen. *Zeitschr. Physiol. Chem.*, **33**: 9–55.

CONWAY, E. J., 1962. Microdiffusion Analysis and Volumetric Error. Fifth Edition. Crosby Lookwood & Son Ltd., London.

DELAUNAY, H., 1931. L'excretion azotée des invertébrés. *Biol. Rev. Cambridge Philos. Soc.*, **6**: 265–301.

LENNOX, F. G., 1940. Distribution of ammonia in larvae of *Lucilia cuprina*. *Nature*, **146**: 269.

MYERS, R. G., 1920. A chemical study of the blood of several invertebrate animals. *J. Biol. Chem.*, **41**: 119–143.

NICHOL, J. A. C., 1960. The Biology of Marine Animals. Sir Isaac and Sons, Ltd.

PROSSER, C. L., AND F. A. BROWN, JR., 1961. Comparative Animal Physiology. Second Edition, Illustrated. W. B. Saunders Co., Philadelphia.

PRZYLECKI, ST. J., 1926. La repartition et la degredation de l'acide urique chez les invertébrés. *Arch. Internat. Physiol.*, **27**: 157–202.

SANZO, L., 1907. Stickstaff-Stoffwechel bei marinen wirbellosen Tieren. *Biol. Centralbl.*, **27**: 479–491.

SMITH, I., 1960. Chromatographic and Electrophoretic Techniques. Volume I. Chromatography. Second Edition. William Heinemann Medical Books Ltd. London.

SOBEL, A. E., A. HIRSCHMAN AND L. BESMAN, 1945. A convenient microtitration method for the estimation of amino acids. *J. Biol. Chem.*, **161**: 99–103.

STADDON, B. W., 1955. The excretion and storage of ammonia by the aquatic larva of *Sialcs lutaria* (Neuropteri). *J. Exp. Biol.*, **32**: 84–94.

VAN DER HEYDE, H. C., 1923. Petites contributions à la physiologie comparée IV Sur l'excretion chez les échinodermes. *Arch. Néerland. de Physiol.*, **8**: 151–160.

INDUCTION OF IMMUNOLOGICAL TOLERANCE BY INTRA-COELOMIC GRAFTS IN THE 4-DAY CHICK EMBRYO

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When cells or antigens are injected into an embryo or a newborn animal a condition of tolerance to the foreign stimulus may be induced. Although the mechanism involved in this induction is not well known, we may entertain two possibilities: (1) that the embryo may react with the cells or antigen, thereby revealing the development of a certain level of immunological competence, and manifest either a tolerant or an immune reaction depending on the dosage (Howard and Michie, 1962; Michie and Howard, 1962). However (2) the host embryo may also nourish the proliferation of the foreign cells and permit the establishment of a chimeric condition which is frequently obtained in tolerant animals (Billingham *et al.*, 1952; Hasek and Hort, 1960; Stone *et al.*, 1965). Although the mechanism is not clear, we may presume that the requirement for immunological competence is not involved in such cases.

In the chick embryo, it is possible to examine these two alternatives as well as elucidate the role of competence in the induction of tolerance by implanting cells or antigens into the coelom of 4-day embryos, well before the onset of competence. Immunologically competent cells, as measured by their ability to elicit a splenomegaly, are not detected until immediately after hatching (Solomon, 1961; Mun *et al.*, 1962). Solomon (1963) reported the sensitization of host lymphocytes as measured by a depressed splenomegaly in the host during the eleventh to seventeenth days of incubation. Ackerman and Knouff (1964) were able to identify certain cell types which may be associated with the production of antibody in the thymus of older 10- and 14-day chick embryos.

We ask first: Can tolerance be induced in the 4-day chick embryo? If so, we may next inquire: Would a greater degree of tolerance be obtained by the exchange of cells or tissue from the same stage of development or by tissue from embryos older than 10 days which may contain immunologically competent cells (Mun, 1965)?

MATERIALS AND METHODS

Two series of experiments were conducted, one at Orono, Maine, and the other at East Lansing, Michigan. In the first series of experiments, the donor tissues were obtained from a White Leghorn (WL) strain obtained from SPAFAS, Inc., Norwich, Conn., which has maintained for 10 or more years a closed flock with continuous inbreeding, but not necessarily with brother-sister mating. The hosts were derived from a cross between a Rhode Island Red male and a Barred Rock female (BR × RIR). In the second series of experiments, donor tissues were obtained from Line 7 embryos and hosts were derived from a cross between Line 15 I and Line 6. These lines have been maintained as independent inbred lines at the Regional Poultry Research Laboratory since 1939 (Crittenden *et al.*, 1964).

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The intracoelomic grafting technique has been previously described by Hamburger (1960) and Dossel (1954). The eggs were incubated for 86 to 96 hours at 99° F. and 85% relative humidity. Embryos which had attained normal developmental stage 21 (Hamburger and Hamilton, 1951) with the allantois almost in contact with the head were selected. A cut was made with a steel needle through both the vitelline membrane and the somatopleure, in the small space between the allantois and the head. The donor tissue, approximately 0.1 mm.^{3}, was then pushed through this opening and into the coelom toward the base of the allantois with a curved and blunted glass needle. The operated eggs with their pointed ends down were placed in the incubator and permitted to hatch.

TABLE I

Mean survival time of homografts in untreated hosts

Host	Donor	No. cases	MST* days	Standard deviation	Range of survival time (days)
BR × RIR	WL (SPAFAS)	53	8.1 ± 0.5	1.89	5–14
151 × 6	Line 7	40	16.8 ± 2.0	6.29	6–31

* Plus and minus 95% confidence limits.

Two to 4 days after hatching, the chicks were divided into groups of four to six, each group being made up of both operated, and unoperated or sham-operated chicks. Each chick in the group then received a skin graft from a donor of the same age and of the same strain as the previous embryonic donor. The donor chick was discarded. The skin grafting technique developed by Cannon and Longmire (1952) was employed. The chicks were randomly numbered and not identified according to treatment. The grafts were read at one- or two-day intervals for the first two to three weeks post-operation and later at weekly intervals. The condition of the grafts in operated animals was compared with autografts as well as grafts in unoperated controls, and the time at which the first signs of rejection appeared was noted. Rejection was usually marked by a sudden darkening of the graft, *e.g.*, from pink or yellow to dark purple or brown, as well as a change in the surface texture, *e.g.*, from a soft, pliable condition to a smooth, hard surface (Polley *et al.*, 1960). After this stage, the hard scab which is formed eventually drops off and the resultant bare area may persist for a variable period of time, until the reappearance of host feathers oriented in the normal direction.

RESULTS

When skin grafts were exchanged between 2- to 4-day hatched chicks from the same WL (SPAFAS) strain, eight out of sixteen grafts (50%) took successfully and remained for more than 20 days. On the other hand, skin grafts from WL (SPAFAS) donors on BR × RIR hosts were all rejected within 15 days (Table 1).

TABLE II

Survival of skin grafts in BR × RIR hosts following intracoelomic grafts of various tissues from WL (SPAFAS) embryos at different stages of development

Treatment	Total no. of cases	No. of grafts surviving 1 to 20 days	No. of grafts surviving more than 20 days
4-day pharyngeal pouch, limb bud or lens	16	16	0
7- or 8-day spleen	25	25	0
12-day spleen	7	7	0
15-day lens	5	5	0
14- to 21-day spleen or thymus	80	71	9 (11%)
None, or sham-operated	53	53	0

Fifteen experiments were conducted in which various tissues from WL (SPAFAS) were implanted into the coelom of BR × RIR embryos. Pooled data from these experiments show that limb buds, lens, or tissues from the region of the pharyngeal pouches 3 and 4 of 4-day embryos, spleen from 7-, 8-, or 12-day embryos and lens from 15-day embryos were not able to induce tolerance in BR × RIR hosts. However, tolerance was induced by spleen and thymus tissues from older 14- to 21-day embryos in 11% of the cases (Table II). Because no striking differences in ability of these two tissues to induce tolerance were observed in these preliminary studies, the data were pooled.

Because of the small percentage of treated animals manifesting tolerance, these experiments were repeated at East Lansing, Michigan, where embryos from highly inbred lines were available. Twenty-two out of 29 (75%) skin grafts between 2- to 4-day Line 7 chicks took successfully and remained more than 50 days. Although the homogeneity with respect to the histocompatibility loci in this particular line is not yet complete (Crittenden *et al.,* 1964), it is greater than that in the WL (SPAFAS) strain (50%). Skin grafts from hatched chicks of Line 7 placed on sham- or saline-operated or unoperated chicks of Lines 15 I × 6 were all rejected within 32 days (Table I).

Ten experiments were conducted in which donor tissues from Line 7 embryos were implanted in the coelom of 4-day 15 I × 6 embryos. Donor tissues were

TABLE III

Survival of skin grafts in 15 I × 6 hosts following intracoelomic grafts of various tissues from Line 7 embryos at different stages of development

Treatment	Total no. of cases	Graft survival time in days		
		1 to 32 days	33 to 50 days	More than 50 days
Intracoelomic grafts of				
(1) 4-day embryonic limb, liver, pouches 3 and 4	64	52	4	8(12.5%)
(2) 9- to 18-day embryonic spleen, thymus, and liver	44	16	10	18(41%)
(3) 1-day hatched chick spleen, thymus	16	10	4	2
Control or sham	40	40	0	0
Autograft 15 I × 6	39	1	0	38(97%)

obtained from limb buds, third and fourth pharyngeal pouches, and liver of 4-day embryos. Spleen, liver and thymus tissues were obtained from 9-day embryos to 1-day hatched chicks. Table III shows that a significantly greater proportion of skin grafts lasting more than 50 days was obtained in chicks receiving intracoelomic grafts from older (9- to 18-day) embryos than from younger (4-day) embryos ($P < 0.005$). Runts disease was observed in a few cases receiving intracoelomic grafts from 1-day hatched chick tissues.

Discussion

Tolerance can be induced in the chick embryo by joining their chorioallantoic membranes on or about the 10th day of incubation (Hasek, Hraba and Hort, 1958) or by cross-transfusion of blood on the 10th to 16th days of incubation (Terasaki, Cannon and Longmire, 1958).

The present data show clearly that tolerance can also be induced in the 4-day chick embryo by implanting various tissues into the coelom. If the initial steps in the mechanism of tolerance induction involve the interaction of the foreign antigen with immunologically competent cells, we may conclude that competent cells are present in the chick embryo at this very early stage of development. However, because immunologically competent cells as measured by other means are not detected until at least after the 10th day of incubation, we may suggest that the foreign donor tissues persist in the host environment and later react with competent host cells as they appear (Mun *et al.*, 1962). On the other hand, the observation that tolerance induction was enhanced by older, more differentiated tissue argues against the notion that tolerance is solely the result of mutual exchange, or persistence of donor tissue or cells in the host environment, and compels us to consider the immediate impact of the foreign cells on the host environment.

There may be several explanations to account for the difference in the ability to induce tolerance:

(1) The older tissues "took" better than grafts from younger donors. Volpe and Gebhardt (1965) observed in the frog that larger homografts, comprising two complete lateral neural folds, survived and persisted indefinitely, while smaller single lateral neural fold homografts were almost invariably eventually rejected. Thus, the older donor grafts with a greater amount of antigen may demonstrate a larger percentage of tolerant cases mainly because of their greater ability to survive in the embryonic environment.

(2) On the other hand if we may assume that both older and younger grafts take equally well, the greater ability of the older tissue to induce tolerance may likewise be due to the amount of antigen. Howard and Michie (1962), Michie and Howard (1962) and others have shown that a larger dose would result in tolerance but a smaller dose of the same antigen would elicit sensitivity. However, we found that intracoelomic grafting of larger pieces of tissues, almost two to three times the usual size (0.1 to 0.3 mm.^3), from either older or younger donors, did not result in the induction of a greater degree of tolerance. The use of large pieces of lens tissue from 15-day chick embryos also did not induce tolerance.

(3) The enhancement of tolerance by the grafts from older donors may also be due to qualitative differences, as well as quantitative differences in antigen supply (Billingham and Silvers, 1962). Ebert (1951) discovered the appearance of

a spleen specific antigen on or about the 18th day of incubation. However, studies on the development of the B blood antigens which are strongly associated with histocompatibility in the chick reveal that they can be detected as early as the 7th day of incubation (L. W. Johnson and W. E. Briles, personal communication).

(4) This leads us to consider another possibility: the impact of immunologically competent cells, which we may find in the 14- to 21-day donor, on the host environment. Jensen and Simonsen (1962) have observed in parabiosis experiments in highly inbred mice, a facilitation of tolerance by the same antigenic stimulus when the parabiont to become tolerant was exposed to a graft-vs.-host reaction from its partner at the same time. The immunologically competent cells may respond to the host antigen by proliferation and the release of greater amounts of donor antigen, thus increasing their effective dosage very rapidly (Billingham and Silvers, 1961, p. 127; see discussion by Burch and Burwell, 1965, p. 271). In the chick, the immunologically competent donor cells may also act to stimulate proliferation of the embryonic host spleen cells contributing to the observed organ enlargement (Danchakoff, 1916; Biggs and Payne, 1961; DeLanney *et al.*, 1962; Mun and Burns, 1965). The role of these host-donor cell interactions in the mechanism of tolerance induction remains to be explored.

We thank Mrs. Nancy McPhee Simpson for expert technical assistance. We are grateful to Dr. James D. Ebert and Mr. Charles Kimmel for helpful suggestions in the preparation of the manuscript. We also thank Dr. B. R. Burmester, Director, U. S. Department of Agriculture, Regional Poultry Research Laboratory, for making both invaluable inbred materials and research facilities available to us at East Lansing, Michigan.

This investigation was supported by grant No. G-22431 from the National Science Foundation to the University of Maine.

SUMMARY

1. In a series of experiments in which non-inbred material was used, 9 out of 80 skin grafts from a White Leghorn strain survived more than 20 days on Barred Rock × Rhode Island Red hosts which had received intracoelomic grafts of spleen and thymus from older (14- to 21-day) embryos of the same donor strain. Hosts which had received intracoelomic grafts of pharyngeal pouches 3 and 4, limb buds and lens from 4-day embryos, or spleens from 7-, 8-, or 12-day embryos or lens tissue from 15-day embryos, rejected skin grafts from the same donor strain within 20 days.

2. When highly inbred material was used, tolerance was induced in Line 15 I × 6 hosts by intracoelomic grafts of limb buds, liver, or pouches 3 and 4 from Line 7 embryos of 4 days. However, a significantly greater degree of tolerance was induced by spleen and thymus tissues from older 9- to 18-day embryos of the same donor strain. The possible impact of near-immunologically competent cells on host cells in the induction of tolerance was considered.

LITERATURE CITED

Ackerman, G. A., and R. A. Knouff, 1964. Lymphocyte formation in the thymus of the embryonic chick. *Anat. Record,* 149: 191–216.

Biggs, P. M., and L. N. Payne, 1961. Pathological changes following the inoculation of chick embryos with adult cells. I. Spleen cells. *Immunology,* 4: 24–37.

Billingham, R. E., and W. K. Silvers, 1961. Quantitative studies on the ability of cells of different origins to induce tolerance of skin homografts and cause runt disease in neonatal mice. *J. Exp. Zool.*, **146**: 113–129.

Billingham, R. E., and W. K. Silvers, 1962. Some factors that determine the ability of cellular inocula to induce tolerance of tissue homografts. *J. Cell. Comp. Physiol., Suppl. 1*, **60**: 183–200.

Billingham, R. E., G. H. Lampkin, P. B. Medawar and H. L. Williams, 1952. Tolerance to homografts, twin diagnosis, and the freemartin condition in cattle. *Heredity*, **6**: 201–212.

Burch, P. R. J., and R. G. Burwell, 1965. Self and not-self: a clonal induction approach to immunology. *Quart. Rev. Biol.*, **40**: 252–279.

Cannon, J. A., and W. P. Longmire, 1952. Studies of successful skin homografts in the chicken. *Ann. Surg.*, **135**: 60.

Crittenden, L. B., L. W. Johnson and W. Okazaki, 1964. Histocompatibility and erythrocyte antigen variability within highly inbred lines of White Leghorns. *Transplantation*, **2**: 362–374.

Danchakoff, V., 1916. Equivalence of different hematopoietic anlages (by method of stimulation of their stem cells). I. Spleen. *Amer. J. Anat.*, **20**: 255–327.

DeLanney, L. E., J. D. Ebert, C. M. Coffman and A. M. Mun, 1962. On the chick spleen: origin; patterns of normal development and their experimental modification. *Carnegie Inst. Washington, Contributions to Embryology*, **37**: 57–85.

Dossel, W., 1954. New method of intracoelomic grafting. *Science*, **120**: 262–263.

Ebert, J. D., 1951. Ontogenetic change in the antigenicity of the chick spleen. *Physiol. Zoöl.*, **24**: 20–41.

Hamburger, V., 1960. A Manual of Experimental Embryology. 221 pages. Univ. of Chicago Press, Chicago, Ill.

Hamburger, V., and H. L. Hamilton, 1951. A series of normal stages in the development of the chick embryo. *J. Morphol.*, **88**: 49–92.

Hasek, M., and J. Hort, 1960. Nonspecific tolerance of grafts and the dissociation of two types of immunity. *Nature*, **186**, 985.

Hasek, M., T. Hraba and J. Hort, 1958. Embryonic parabiosis and related problems. *Ann. N. Y. Acad. Sci.*, **73**: 570–574.

Howard, J. G., and D. Michie, 1962. Induction of transplantation immunity in the newborn mice. *Transpl. Bull.*, **29**: 91–96.

Jensen, E., and M. Simonsen, 1962. Induced tolerance after parabiosis: apparent facilitation of tolerance by a simultaneous graft-*versus*-host reaction. *Ann. N. Y. Acad. Sci.*, **99**: 657–662.

Michie, D., and J. C. Howard, 1962. Transplantation tolerance and immunological immaturity. *Ann. N. Y. Acad. Sci.*, **99**: 670–679.

Mun, A. M., 1965. Ontogeny of tolerance induction in the chick embryo. *Amer. Zool.*, **5**: 252.

Mun, A. M., and E. R. Burns, 1965. Donor-host cell interaction in homologous splenomegaly in the chick embryo. *Biol. Bull.*, **127**: 467–477.

Mun, A. M., P. Tardent, J. Errico, J. D. Ebert, L. E. DeLanney and T. S. Argyris, 1962. An analysis of the initial reaction in the sequence resulting in homologous splenomegaly in the chick embryo. *Biol. Bull.*, **123**: 366–387.

Polley, C. R., A. E. Grosse and J. V. Craig, 1960. A skin grafting technique for use in genetic studies with chickens. *Transplantation Bull.*, **7**: 425–428.

Solomon, J. B., 1961. The onset and maturation of the graft *versus* host reaction in chickens. *J. Embryol. Exp. Morphol.*, **9**: 355–369.

Solomon, J. B., 1963. Actively acquired transplantation immunity in the chick embryo. *Nature*, **198**: 1171–1173.

Stone, W. H., R. G. Cragle, E. W. Swanson and D. G. Brown, 1965. Skin grafts: delayed rejection between pairs of cattle twins showing erythrocyte chimerism. *Science*, **148**: 1335–1336.

Terasaki, P. I., J. A. Cannon and W. P. Longmire, Jr., 1958. The specificity of tolerance to homografts in the chickens. *J. Immunol.*, **81**: 246–252.

Volpe, E. P., and B. M. Gebhardt, 1965. Effect of dosage on the survival of embryonic homotransplants in the Leopard Frog, *Rana pipiens*. *J. Exp. Zool.*, **160**: 11–28.

PERSISTENT, VERTICAL-MIGRATION RHYTHMS IN BENTHIC MICROFLORA. VI. THE TIDAL AND DIURNAL NATURE OF THE RHYTHM IN THE DIATOM HANTZSCHIA VIRGATA[1]

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During ebb tide in certain intertidal mud and sand-flats, irregular areas of the exposed substratum become green or golden brown in color. With the return of the flooding tide—or often just prior to its return—the color fades and disappears. Microscopic examination of these sediments reveals that the color may be due to a superficial accumulation of enormous numbers of protozoans, small metazoans, or more commonly, single-celled algae. These organisms dwell in the sediments during tidal inundation and move up onto the surface sands during tidal exposure—a behavior pattern called a vertical-migration rhythm. Dinoflagellates (Herdman, 1924), euglenoids (Bracher, 1919; Palmer and Round, 1965), a chrysomonad (Fauré-Fremiet, 1950), several species of diatoms (Fauvel and Bohn, 1907; Aleem, 1950; Callame and Debyser, 1954; Round and Palmer, 1966), and a zooxanthellae-containing planarian (Gamble and Keeble, 1903) are all known to undergo these tide-associated rhythms in vertical migration. At times these organisms are present in such great numbers that one investigator (Herdman, 1924, p. 59) observed that ". . . the diatoms were so abundant on the surface that their photosynthetic activity was distinctly *audible* as a gentle sizzling . . . and the sand was frothy with bubbles of gas, presumably oxygen given off by them."

The rhythmic behavior of a few of these organisms has been studied in the laboratory and found to persist in natural day-night conditions but in the absence of the tide (Fauvel and Bohn, 1907; Bracher, 1919; Herdman, 1924; Fauré-Fremiet, 1950, 1951), and in constant conditions, *i.e.*, constant temperature, continuous illumination of a constant intensity, and no tides (Palmer and Round, 1965; Round and Palmer, 1966). Our studies (*loc. cit.*) have revealed an interesting and unexpected aspect of vertical-migration rhythms, namely, that in constant conditions the rhythms of two species of *Euglena* and eight species of diatoms are *diurnal*, rather than *tidal*, *i.e*, the 24.8-hour period of the rhythm—as displayed in nature—is not expressed in the laboratory; instead, the persistent rhythm has a 24-hour period. These data, combined with certain field studies by other investigators (*e.g.*, Perkins, 1960), suggest that possibly all overt tidal vertical-migration rhythms might actually represent underlying 24-hour rhythms which are entrained and thus transformed by the tides in nature. Alone among the modern studies in contradiction of this supposition, is the work of Fauré-Fremiet (1951) on the vertical-migration rhythm of the diatom *Hantzschia am-*

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[1] This work was supported by National Science Foundation grants GB-5045 to JDP, and GB-4509 to the Marine Biological Laboratory.

phioxys. He reports (p. 173) that when *Hantzschia*-bearing sand samples were returned to the laboratory and ". . . exposed to diffuse light from the window, on succeeding days (the longest period of observation being six days), the [*Hantzschia*] re-appeared on the surface of the sand at the same time as low tide in their natural habitat." He used only the color change of the sand as an indication of whether or not the cells were on the surface and stresses the lack of precision of this type of observation. Because his work stands as an exception to our original hypothesis, it has stimulated us to re-examine the vertical migratory behavior of this organism in greater detail and using quantitative methods. Both field and laboratory studies were carried out.

This diatom inhabits the intertidal sand-flats of Barnstable Harbor, Cape Cod, Mass., and was previously identified in the paper of Fauré-Fremiet (1951) as *H. amphioxys.* We have compared the diatom with collections held at the Philadelphia Academy of Sciences and the British Museum and find that it is *H. virgata* var. *intermedia* (Grun.).[2] During the summer months it tends to be the dominant species of an algal community containing the diatoms *Amphora, Navicula, Amphiprora, Pleurosigma,* and *Nitzschia;* the dinoflagellate *Amphidinium;* the euglenoids, *Euglena* and *Trachelomonas;* and the cyanophyceans *Chroococcus, Merismopedia* and *Oscillatoria.* Preliminary studies indicate that all these subdominants also undergo vertical-migration rhythms in the field and the laboratory.

METHOD

In order to obtain quantitative estimates of cell concentrations on the surface sediments at any one time, a method previously described in the literature (Palmer and Round, 1965) was employed. In brief, just as the ebbing tide uncovered the sampling station, numerous small pieces (9 mm.^{2}) of ordinary microscope-lens-cleaning tissue were placed on the sediment surface. The *Hantzschia,* in their migratory ascent to daylight, moved up through the sediments and into the interstices of the paper. Tissues were then periodically removed from the sediment during tidal exposure, the diatoms washed out in a drop of water on a microscope slide, and their numbers counted. Replicate samples were taken and averaged.

In order to collect cells for study in the laboratory, 10-mm. lengths of glass tubing (35 mm. in diameter) were inserted into the sediment and removed with a core of *Hantzschia*-bearing sand within them. These cores, still retained within the glass rings, were placed in small Petri dishes and returned to the Marine Biological Laboratory at Woods Hole, Mass., where water was added to the moat-like space between the outside of the glass ring and the inner wall of the Petri dish. The samples were kept in Precision Scientific Incubators at a constant temperature of 18° C. and overhead illumination of 110 foot-candles from Westinghouse, 15-watt, cool-white fluorescent tubes. The cells were maintained in alternating light-dark photoperiods (symbolized at L:D) with the light on between 0530 and 2000 hours (the approximate time between sunrise and sunset), or in continuous illumination (L:L). It should be pointed out, however, that inherent in vertical-migration rhythms is a periodic sojourn beneath the sand surface, placing the organisms into semi-darkness. This obviously tends to negate the desired effect of the overhead experimental light regime. The laboratory populations were also sampled with the lens-paper technique and the average of 3–6 samples used for each cell count. Because this technique unavoidably reduces the size of the sample populations, during long-term observations, sampling on some days was intentionally omitted.

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[2] We wish to thank Dr. R. Patrick and Mr. R. Ross for their help and the loan of type material.

RESULTS

Field observations

The sampling station on the Barnstable Harbor sand-flats is uncovered by the tide for an average duration of 4.5 hours once every 12.4 hours. Field observations were made at intervals during the summers of 1965 and 1966 and were timed so that the presence of the cells on the surface during morning, midday, and evening low tides could be described and compared.

It was found that when low tide straddles the time of sunrise the cells do not appear on the surface until shortly after the time of sunrise. Once they begin to appear, their numbers rapidly increase to a maximum value which then remains relatively constant until about 30–60 minutes before the return of high tide, at which time the cells begin to re-burrow back into the substratum. A representative curve is described in Figure 1.

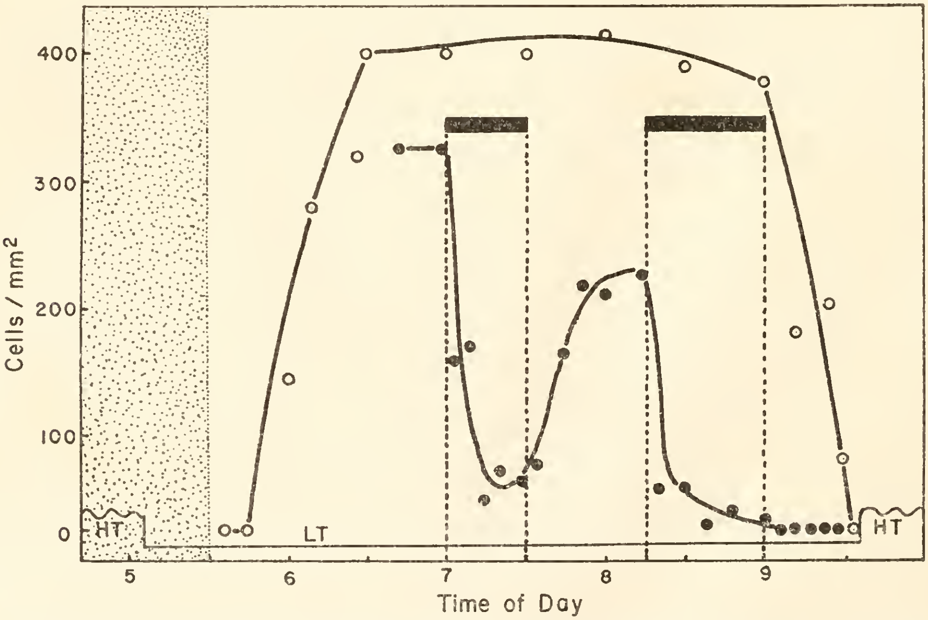

FIGURE 1. Field observations of the vertical-migration rhythm in *Hantzschia*. Wavy lines (HT) represent the times of high tide. Straight line (LT) subtending and connecting consecutive wavy lines indicates time of low tide. The time of sunrise is represented by the boundary between stippled and unstippled areas. The shaded horizontal bars supported by dashed lines signify periods when the sands were covered by opaque canisters. See text for further description.

During midday exposures, the cells begin to appear on the surface 15–30 minutes after the tidal water recedes, increase in number to a fairly constant density which is maintained for about three hours, and then commence to re-burrow about 30 to 60 minutes in advance of the incoming tide. (Re-burrowing in *anticipation* of actual return of flood tide is a common feature of vertical-migration rhythms [Callame and Debyser, 1954; Palmer and Round, 1965; Round and Palmer, 1966].) By the time of inundation only the remnants of the densest patches remain on the surface and these cells re-burrow within 15 minutes after being submerged. A small fraction of the cells is often washed away. Measurements of the sediment water content during a tidal exposure showed that on a bright, windy day, the water content drops as much as 14% below the value obtained just after exposure. Up to one hour before the actual reflooding of the area—and simultaneous with the time the cells begin to re-burrow—the interstitial water content of the sand begins to increase steadily. This may well be the stimulus which initiates re-burrowing in apparent anticipation of the return of high water. As will be shown later, however, re-burrowing is under the control of a biological clock and the cells need no external stimulus if this kind to re-burrow.

As late afternoon low tides approach the time of sunset, the diatoms do not remain on the surface for the duration of low water, but instead re-burrow slightly before sunset. When the sand flats are first exposed at 1630 hours, or later, the cells never appear on the surface.

The diatoms never appear on the surface at night and they can be kept from emerging on the surface during daylight by artificially darkening the sediments with opaque canisters. Similarly, cells already on the surface can be made to re-burrow by artificial darkening. This is seen in Figure 1, where the cells were artificially darkened between 0700 and 0730 and again between 0845 and 0900. After the first darkening the cells returned to the surface; after the second, they did not, but at this time the rest of the population was also in the process of re-burrowing. The upper curve in this figure represents untreated cells in an adjacent patch and thus acts as a control for the darkening experiments.

Laboratory studies

The rhythmic behavior of *Hantzschia* was first studied in constant light and temperature. Under these conditions the rhythm was found to persist for as long as eleven days. A representative experiment is seen in Figure 2. While nighttime values were not determined for this particular set of data, numerous other all-night observations have adequately demonstrated that the cells never appear on the surface at night, even when samples are maintained in L:L. The approximate times of low tide in nature are indicated for each day, and show that the cells in the laboratory appear on the surface in approximate synchrony with those in Barnstable Harbor—strongly suggesting that the rhythm is actually tidal. However, the possibility exists that the rhythm may actually be one with a fundamental period of 24 hours, which—as is common with most persistent rhythms—has become circadian in constant conditions, and by chance, has a period of 24.8 hours. To test this possibility, samples were placed in L:D, a condition which restricts solar-day rhythms to their fundamental 24-hour period. Figure 2 shows that the cells continue to appear on the surface later each day and again in approximate synchrony

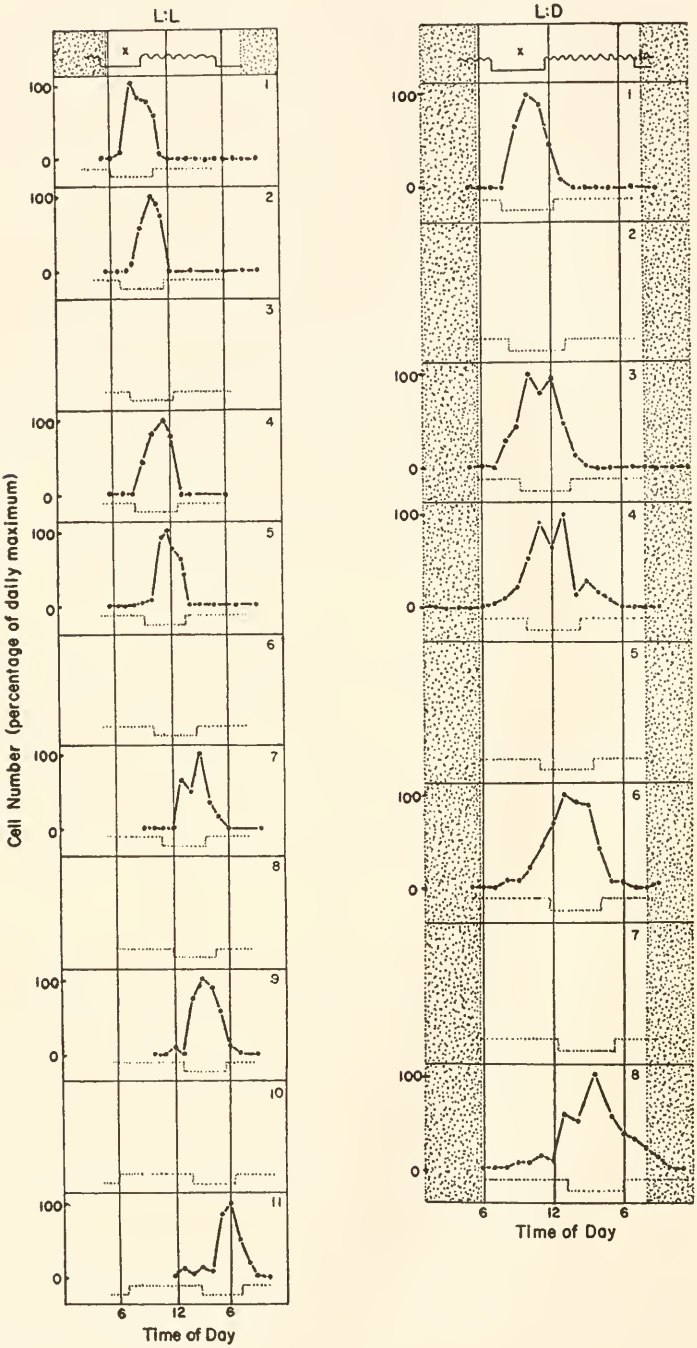
L:L
L:D
x
Cell Number (percentage of daily maximum)
100
0
1
2
3
4
5
6
7
8
9
10
11
6
12
6
Time of Day

FIGURE 2.

with the tidal exposure in nature. The persistent rhythm is indeed a lunar-day rhythm.

The 12.4-hour interval between successive low tides is such that in the summer, when an afternoon low tide approaches the time of sunset, the following morning low tide begins to overlap with sunrise. Therefore, twice each month, there are a few days when the sand flats are exposed to sunlight twice a day: once in the morning and again in the afternoon. As the afternoon exposure approaches dusk, the diatoms abandon this phase of the rhythm and now appear on the surface during the early morning low tide. This rather drastic change in phase was studied in the laboratory.

Cells were collected during a mid-afternoon low tide and placed in L:D in the incubators. Sampling began the next day and, as seen in Figure 3, the cells came up strongly in the late afternoon. On the second day the afternoon peak virtually collapsed, and by the third day the cells now appeared in the morning. The same observations were carried out in L:L and, quite remarkably, the same change in phase occurred. Both the observations in L:D and L:L have been repeated several times with the same results.

Discussion

Clock control of the rhythm

Persistent, tidal rhythms have been previously described for a variety of physiological functions, *e.g.,* oxygen consumption in crabs (Brown *et al.,* 1954) and snails (Sandeen *et al.,* 1954); spontaneous locomotor activity in crabs (Bennett *et al.,* 1957; Naylor, 1958; Palmer, 1966), amphipods (Enright, 1962; Morgan, 1965), and fish (Gibson, 1965), and in filtration rate in mussels (Rao, 1954). These rhythms may be described as tidal rhythms (*i.e.,* rhythms with periods of 12.4 hours), or better, as *bimodal* (or *biphasic*) lunar-day rhythms with periods of 24.8 hours. When studied in relation to a 24-hour-day scale, the dual peaks of the lunar-day rhythm advance at a rate of 50 minutes/cycle across the solar day.

The overt lunar-day rhythm in *Hantzschia* differs considerably from the above rhythms in two major ways. First, the rhythm is *unimodal, i.e.,* the cells appear on the surface only once every 24.8 hours. Secondly, the single maximum scans across the hours corresponding to daylight at a tidal rate of 50 minutes/day and then, in a matter of just a few days, rephases back to the morning hours again. Any model derived to explain the rhythm in *Hantzschia* must take into account these two unique properties of the rhythm.

The curves obtained in L:D, shown in Figure 3, indicate that when the suprasurface phase of the rhythm reaches the dark portion of the imposed photoperiod the rhythm rephases to the morning hours, suggesting that the times of "light off"

FIGURE 2. Persistence of the vertical-migration rhythm in constant light (L:L) and in alternating light-dark period (L:D). In both conditions the rhythm displays a period of about 24.8 hours. Consecutive days run from top to bottom. Stippling indicates dark periods. X = time of collection of samples. State of tide on day of collection symbolized as in Figure 1. Depressions in dotted lines represent times of low tide in nature on days when rhythm was studied in the laboratory. For ease in comparison the data are expressed in percentages (the highest cell count in each cycle was designated as 100 and all other values as percentages of this). In no case was 100% less than 2.9×10^3 cells/cm.^{2}.

and "light on" act as guideposts for the extreme phase relationships of the rhythm. By way of analogy, the scanning movements of the single peak across the day can be likened to the movement of a typewriter carriage, which slowly and systematically—one letter at a time—moves across the instrument to the far carriage stop, and is then rapidly swept back to the starting margin to begin another journey. The carriage stops, which dictate the extent of movement of the carriage, could be

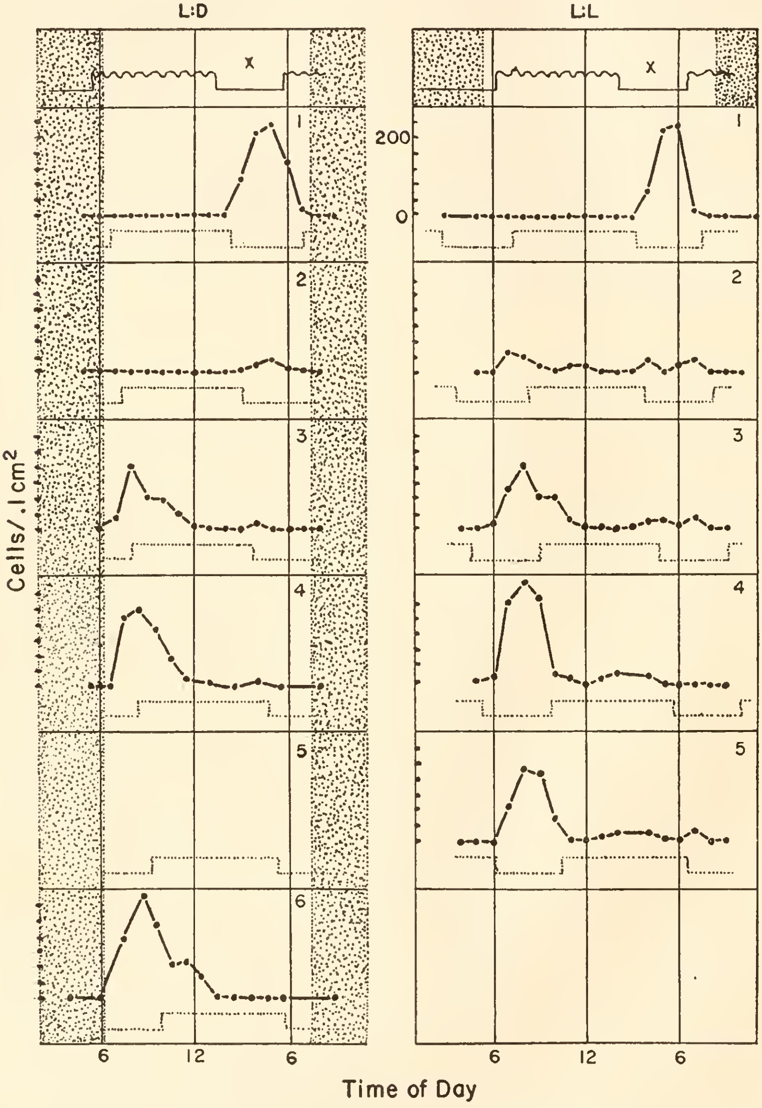

FIGURE 3. The phase change of the persistent vertical-migration rhythm in alternating light-dark periods (L:D) and in constant light (L:L). Symbols the same as Figure 2. Ordinate scale for all days given in upper right hand column.

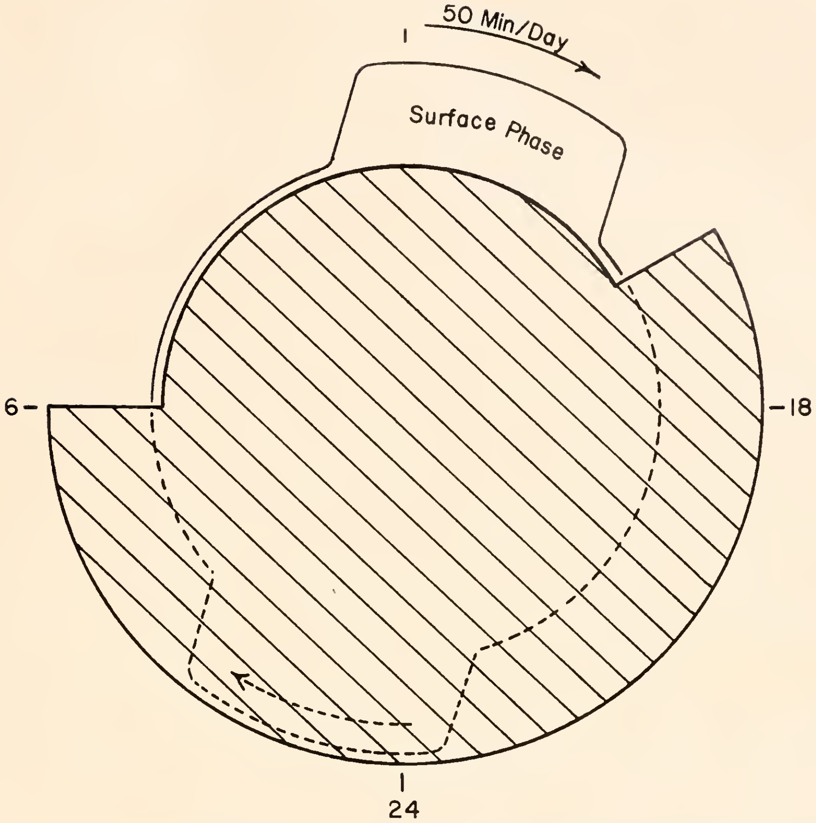

Figure 4. Diagrammatic representation of the interaction of a 24.8-hour bimodal vertical-migration rhythm (here represented as a disk with opposing bulges; each bulge signifying the surface phase of the rhythm) and a 24-hour suppression-expression rhythm (represented as an incomplete disk superimposed over the lunar-day rhythm). The shaded area of the disk is that part of the 24-hour rhythm that suppresses the night-time phase of the migration rhythm, and the open segment the part that allows the expression of the daytime supra-surface phase. Because the supra-surface phase of the lunar rhythm occurs 50 minutes later each day it eventually falls under the influence of the suppressive portion of the solar-day rhythm. As this phase is inhibited the unexpressed early morning phase is expressed. The net result is an *apparent* rephase of the migration rhythm.

likened to the times of "light on" and "light off" in the rephasing of the rhythm. However, the analogy breaks down when the rhythm is re-examined in L:L as no such obvious "stops" were then present, yet the same apparent rephase took place. As a consequence, we prefer to adopt a working hypothesis based on the presence of two interacting clock systems. One is a lunar-day clock which, of course, measures periods of 24.8 hours. This clock controls a vertical-migration rhythm characterized by *two* supra-surface phases, 12.4 hours apart. This rhythm is represented diagrammatically in Figure 4, by a rotating disk with diametrically opposed, conspicuous bulges. Each of these lateral protrusions represents a supra-surface phase of the rhythm and therefore each has a width equal to 4.5 hours. Coupled to the lunar-day clock is a solar-day clock which measures periods of 24 hours. This horologue controls a rhythm which is characterized by two alternating phases: one which suppresses the night-time supra-surface phase of the bimodal vertical-migration rhythm, and a second phase which permits the expression of the migration rhythm in the daytime. The action of this rhythm is represented in Figure 4, by a partially shaded stationary disk superimposed over the disk representing the lunar-day rhythm. The shaded section represents the suppressive role of this rhythm and the open segment or "window" represents the portion in which the tidal rhythm is not inhibited. The size of the "window" was determined by field observations of the hours of daylight during which the cells appeared on the surface.

By means of such a dual mechanism, as the expressed phase of the lunar-day rhythm occurs progressively later each day (50 minutes/day), it eventually coincides with the suppressive phase of the solar-day clock and is inhibited. Concurrent with this event, the opposite peak of the migration rhythm moves into the "window" and is now expressed. The net result is an *apparent* rephase of the rhythm from afternoon to morning hours.

The feasibility of such a hypothesis is strengthened by the studies of Naylor (1958), Barnwell (1963), Chandrashekaran (1965), and Palmer (1966), which all demonstrate that it is a very common feature for organisms which display persistent lunar-day rhythms to have a solar-day clock system associated with, and modifying, the lunar rhythms. Enright's conclusion (1963), that a single organismic process does not have simultaneous tidal and solar-day components, has now been shown to be premature; it was based on his interpretation of earlier work (Bennett *et al.*, 1957; Naylor, 1958) and by his own work on an organism which possessed only a tidal rhythm (Enright, 1962). Actually, in intertidal organisms, the co-existence of solar-day and lunar-day components in a particular rhythmic function is a commonly encountered pattern.

Role of light

The importance of light in the expression of the vertical-migration rhythm of *Hantzschia* manifests itself in a variety of ways: (1) the cells never appear on the surface during night-time low tides; and when low tides straddle the time of light and darkness, the cells appear on the surface only during the illuminated portion of the tidal exposure, (2) cells may be prevented from appearing on the surface by artificial darkening, and (3) cells already on the surface can be made to reburrow by artificial darkening. It is therefore quite apparent that light is necessary to bring the cells to the surface and to hold them there for some critical time. However, light does not always have an attractive effect, for in L:L in the laboratory, the cells do not come to the surface during the times corresponding to night, or daytime high tides. Reasoning deductively, this certainly indicates that the *Hantzschia* must undergo a rhythmic change in responsiveness to light and this rhythm must be of fundamental importance in their migratory behavior. While no systematic studies have yet been made on the existence of a persistent tidal rhythm in phototaxis in *Hantzschia,* field observations by Fauré-Fremiet (1951) and Palmer (1960) have demonstrated a sign reversal in the phototactic response of this diatom during the supra-surface phase of its rhythm. The organisms were found to respond positively to light during the initial and mid-portions of their stay on the sediment surface and then become indifferent or negative to light just before the return of the tide. A persistent rhythm in phototaxis is known for another unicell, *Euglena* (Pohl, 1948).

Other environmental factors also contribute to the migratory movements of the diatom. Inundation by high tide water is of paramount importance in the expression of the rhythm (Palmer, 1960), and the fact that the diatoms re-burrow when artificially darkened (burrowing being a specific *directional* movement) indicates that geotactic orientation must also be important.

Adaptive nature of the rhythm

It has been tacitly assumed by past investigators that a vertical-migration rhythm represents a highly adaptive relationship with the environment. It is supposed that these sand-dwelling organisms move out onto the surface in order to undergo maximum photosynthetic activity during the daytime, and then re-burrow to avoid being washed away by the returning tide (Ganapati *et al.*, 1959). Certainly some of the non-conformers who do not re-burrow before the flooding tide sweeps over them are often seen to be washed away, thus supporting the latter half of the above contention. However, Taylor and Palmer (1963) have described the photosynthetic light-saturation curve for the benthic microflora community on Barnstable—*Hantzschia*, of course, being a prominent member—and these results demonstrate that sufficient light penetrates through the upper 1.5 mm. of sediment to enable the cells to photosynthesize at above 90% of their maximum capacity. Full sunlight is well above the optimum and actually inhibits photosynthesis somewhat. Quite clearly, then, it is unnecessary for the cells to "risk" a journey onto the surface—and the possibility of being washed away—in order to undergo efficient photosynthesis. It may be that the response is just primarily a phototactic one, the adaptive significance of which is less obvious.

We wish to thank Gary Tabor for technical assistance with the project.

SUMMARY

1. The diatom, *Hantzschia virgata*, appears on the surface sands of Barnstable Harbor, Mass., during *daytime* low tides. Surface accumulations of this organism reach such concentrations that the sand takes on a golden-brown color. As the tide returns the cells re-burrow into the sand.

2. The cells can be prevented from emerging onto the surface sands at low tide by artificially darkening the area with an opaque covering just as the tide recedes. Cells already on the surface can be made to re-burrow by similarly placing them in darkness.

3. The vertical-migration rhythm will persist in the laboratory in constant illumination, constant temperature, and away from the influence of the tide for as long as eleven days. During this time the cells remain in approximate synchrony with the feral cells in nature.

4. In nature, when the times of low tide approach sunset, the cells rephase their rhythm to the early morning hours of daylight. Cells collected during late afternoon low tides and returned to L:D or L:L in the laboratory, undergo a similar rephasing in an interval of just three days.

5. To explain the various unique properties of this rhythm, it is postulated that the rhythm is a manifestation of an interacting dual-clock system: a lunar-day clock which measures periods of 24.8 hours and is responsible for a bimodal migration rhythm; and a solar-day clock responsible for the suppression of the night-time supra-surface phase of the migration rhythm.

LITERATURE CITED

Aleem, A. A., 1950. The diatom community inhabiting the mud flats at Whitstable. *New Phytol.*, **9**: 174–188.

Barnwell, F. H., 1963. Observations on daily and tidal rhythms in some fiddler crabs from equatorial Brazil. *Biol. Bull.*, **125**: 399–415.

Bennett, M. F., J. Shriner and R. A. Brown, 1957. Persistent tidal cycles of spontaneous motor activity in the fiddler crab, *Uca pugnax*. *Biol. Bull.*, **112**: 267–275.

Bracher, R., 1919. Observations on *Euglena deses*. *Ann. Bot.*, **33**: 93–108.

Brown, F. A., Jr., M. F. Bennett and H. M. Webb, 1954. Persistent daily and tidal rhythms of O_2-consumption in fiddler crabs. *J. Cell. Comp. Physiol.*, **44**: 477–506.

Callame, B., and J. Debyser, 1954. Observations sur les mouvements des diatomées à la surface des sédiments marins de la zone intercotidale. *Vie Milieu*, **5**: 242–249.

Chandrashekaran, M. K., 1965. Persistent tidal and diurnal rhythms of locomotory activity and oxygen consumption in *Emerita asiatica*. *Zeitschr. vergl. Physiol.*, **50**: 137–150.

Enright, J. T., 1962. The tidal rhythm of activity of a sand beach amphipod. *Zeitschr. vergl. Physiol.*, **46**: 276–313.

Enright, J. T., 1963. Endogenous tidal and lunar rhythms. *Proc. Int. Congr. Zool.*, **4**: 355–359.

Fauré-Fremiet, E., 1950. Rhythme de marée d'une *Chromulia psammophile*. *Bull. Biol. France Belgique*, **84**: 207–214.

Fauré-Fremiet, E., 1951. The tidal rhythm of the diatom *Hantzschia amphioxys*. *Biol. Bull.*, **100**: 173–177.

Fauvel, P., and G. Bohn, 1907. Le rhythme des marées chez les diatomées littorales. *C. R. Séanc. Soc. Biol.*, **62**: 121–123.

Gamble, F. W., and F. Keeble, 1903. The bionomics of *Convoluta roscoffensis*, with special reference to its green cells. *Proc. Roy. Soc. London, Ser. B*, **72**: 93–98.

Ganapati, P. N., M. V. Lakshmana Rao and D. V. Subba Rao, 1959. Tidal rhythms of some diatoms and dinoflagellates inhabiting the intertidal sands of Visakhapatnam Beach. *Curr. Sci.*, **11**: 450–451.

Gibson, R. N., 1965. Rhythmic activity in littoral fish. *Nature*, **207**: 544–545.

Herdman, E. C., 1924. Notes on dinoflagellates and other organisms causing discoloration of the sand at Port Erin. *Proc. Trans. Liverpool Biol. Soc.*, **38**: 58–63.

Morgan, E., 1965. The activity rhythm of the amphipod *Corophium volutator* (Pallas) and its possible relationship to changes in hydrostatic pressure associated with the tides. *J. Anim. Ecol.*, **34**: 731–746.

Naylor, E., 1958. Tidal and diurnal rhythms of locomotory activity in *Carcinus meanas* (L). *J. Exp. Biol.*, **35**: 602–610.

Palmer, J. D., 1960. The role of moisture and illumination on the expression of the rhythmic behavior of the diatom, *Hantzschia amphioxys*. *Biol. Bull.*, **119**: 330.

Palmer, J. D., 1966. Daily and tidal components in the persistent rhythmic activity of the crab, *Sesarma*. *Nature* (in press).

Palmer, J. D., and F. E. Round, 1965. Persistent, vertical migration rhythms in benthic microflora. I. The effect of light and temperature on the rhythmic behavior of *Euglena obtusa*. *J. Mar. Biol. Assoc.*, **45**: 567–582.

PERKINS, E. J., 1960. The diurnal rhythm of the littoral diatoms of the River Eden estuary, Fife. *J. Ecol.*, **48**: 725–728.

POHL, R., 1948. Tagesrhythmus im phototaktischen Verhalten der *Euglena gracilis*. *Zeitschr. Naturf.*, **3b**: 367–374.

RAO, K. P., 1954. Tidal rhythmicity in the rate of water propulsion in *Mytilus*, and its modifiability by transportation. *Biol. Bull.*, **106**: 353–359.

ROUND, F. E., AND J. D. PALMER, 1966. Persistent, vertical-migration rhythms in benthic microflora. II. Field and laboratory studies of diatoms from the banks of the River Avon. *J. Mar. Biol. Assoc.*, **46**: 191–214.

SANDEEN, M. I., G. C. STEPHENS AND F. A. BROWN, JR., 1954. Persistent daily and tidal rhythms of oxygen consumption in two species of marine snails. *Physiol. Zoöl.*, **27**: 350–356.

TAYLOR, W. R., AND J. D. PALMER, 1963. The relationship between light and photosynthesis in intertidal benthic diatoms. *Biol. Bull.*, **125**: 395.

STUDIES ON DOMECIA ACANTHOPHORA, A COMMENSAL CRAB FROM PUERTO RICO, WITH PARTICULAR REFERENCE TO MODIFICATIONS OF THE CORAL HOST AND FEEDING HABITS

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In the summer of 1965, a survey was made of the fauna of living portions of *Acropora* colonies at La Parguera, on the south coast of Puerto Rico. Collections and underwater observations were made in shallow depths (0–10 feet) at the west end of Enrique Reef. This region has good coral growth but less wave action than the southern, seaward face of the reef (Almy and Carrión-Torres, 1963). Contrary to my expectations, the xanthid crab *Domecia acanthophora* (Desbonne and Schramm) was the only commensal found. Studies were made on the biology of this animal and on its relationship with the host coral.

I am grateful to Dr. P. W. Glynn, Acting Director of the Institute of Marine Biology of the University of Puerto Rico at Mayagüez for reading portions of the manuscript and for the assistance which he and the staff of the Institute offered during my stay in Puerto Rico. Dr. John Garth, of the Allan Hancock Foundation, kindly donated specimens of *Domecia hispida* from the Galapagos Islands.

Methods

The coral to be collected was covered as completely as possible with one or more cloth bags while still in place, then broken off and returned to the laboratory for study. Placing the coral inside bags was awkward but necessary since many of the crabs would have escaped if the coral had simply been lifted from the water. At the laboratory dock, the pieces of coral were weighed and carefully examined while the cloth bags were rinsed in a bucket of water which was poured through a fine mesh screen. The crabs found on each colony were collected and measured. In addition, living crabs were studied both in nature and in the laboratory.

Observations and Conclusions

Domecia acanthophora is a small crab with a mottled brown and cream carapace. Of 180 specimens examined, 162 were above 5.0 mm. in carapace breadth and easily identifiable as to sex, 58 being males and 104 females. The largest male had a carapace breadth of 15.0 mm. while the mean for males was 8.2 mm. For females the comparable figures were 14.0 mm. and 8.4 mm. The smallest ovigerous female had a carapace breadth of 5.6 mm.

The host coral

Three species of *Acropora* are known from the Caribbean (Wells, 1956) and all were found at Enrique Reef (Fig. 1). *Acropora cervicornis* (Lamarck) has branches up to 25 mm. in diameter and grows in large loosely branching colonies which would appear to offer the crabs very little shelter. About ten colonies were examined carefully in the water and crabs were indeed found to be quite scarce, with occasional individuals occurring at a fork or some other site which offered a little protection.

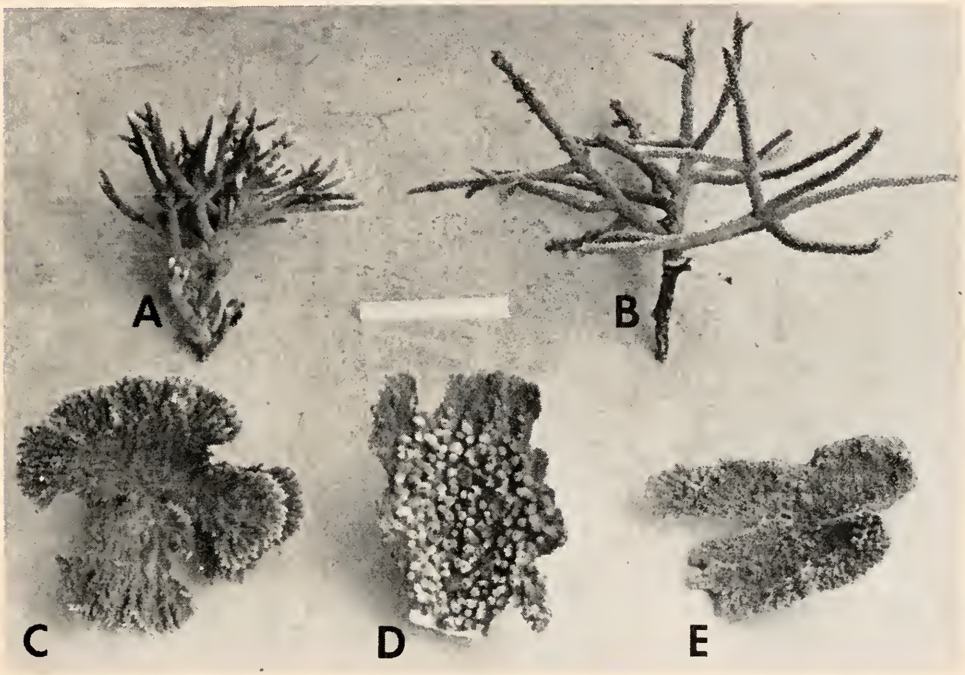

FIGURE 1. A, *Acropora prolifera*. B, *Acropora cervicornis*. C–E, *Acropora palmata*. C, A colony with considerable peripheral branching. D, A colony with considerable algal-induced vertical branching. E, A colony with little peripheral branching.

Acropora prolifera (Lamarck) is similar to the above species but has thinner, more closely spaced branches and forms thickets offering considerable shelter. The crabs seemed to have a patchy distribution, as certain regions of coral contained four or five crabs in a space of 25 cm.^{3}, while much larger and apparently equally suitable regions had none at all.

Colonies of the third species, *Acropora palmata* (Lamarck), are much more massive than those of the preceding two, and typically consist of a central trunk with a number of flattened, horizontal sheets of coral spreading out laterally. These colonies range up to 6 feet in height and are very abundant on the La Parguera Reefs. The peripheral portions of the colony are usually branched to varying degrees but as new outward growth occurs, the spaces between the older branches are filled in, forming a solid, central plate of coral. The peripheral branching was most delicate and extensive in small colonies in about eight feet of water in the channel off the western edge of the reef and seemed least developed in colonies on the seaward face of the reef. This observation is similar to that of Almy and Carrión-Torres (1963) who found small finger-like peripheral branches in colonies growing in the back-reefs. With regard to the central coral plates which compose the bulk of the colony, many are quite smooth while others show varying degrees of diagonal or vertical branching on the upper surface. These branches are generally quite short. An interesting type of vertical branch has a tuft of algae in the center (Fig. 1D). This extends down to the base of the branch and thus it seems likely that the plate was damaged in some way, allowing the algae to settle, and that the coral has grown up around it. On one occasion a coral plate was seen which contained round white spots where the coral tissue had been removed. These spots were grouped in a manner similar to that often found for the algae-tipped branches and were identified by Dr. Glynn as the work of the polychaete *Hermodice carunculata* (Pallas) (Marsden, 1962; Glynn, 1963). It would seem that algae could settle easily on the exposed spots and that polychaete predation may thus contribute to branch formation.

Acropora palmata contains relatively more specimens of *Domecia acanthophora* than do the preceding two coral species and was studied the most intensively. On examination it is seen that the great majority of crabs are not merely sheltering among natural features of the colony but instead are inhabiting structural deformations of the living coral, which, for lack of a better name, will be called "resting places." The term gall should perhaps be reserved for more regular deformations than those shown here. These resting places (Fig. 2) can be divided into three general types: crevices, pits and spaces between vertical branches. I believe that all of these types result from the response of the coral to the continuing presence of a crab.

A crevice is the most inclusive category and is simply a space between a branch and the adjacent coral. When a crab is removed from its crevice, it is seen that the coral has grown away from and around the crab, forming a shelter for it. In addition, the corallites in the region touched by the crab are either thickened and rounded off or absent altogether. Crevices are most common in colonies where much natural peripheral branching occurs and where they can be seen in all stages of development. They can also be found under diagonal branches. I feel that a crevice originates when a young crab settles in an available space and stays there long enough for the coral to be modified by its presence. As the coral continues to grow and the spaces between branches begin to be filled in, the two sides of the crevice may grow so that the crab comes to occupy a slit extending down into the colony. In some cases, the sides of the crevice will unite beyond the crab leaving a space extending from one side of the colony to the other.

Another and common alternative is that the crab will be surrounded on all sides but one by growing coral and the crevice then will be converted into a blind pit. The coral inside a pit is, of course, dead, while the opening is surrounded by a raised lip of corallite-free, living coral. These pits may be quite shallow or may extend well down inside the coral skeleton, depending on the degree to which the opening has remained in the region of active coral growth. Since Vaughan (1915) has reported that *A. palmata* may increase in diameter as much as 95 mm. a year, it would seem that a well formed pit could develop in six months or less and that a rudimentary crevice could develop in a matter of weeks.

Crabs are also found sheltering between certain vertical branches and these spaces too are found to be modified. They are characterized by thickened corallites and a slightly deformed growth of the branches involved.

It can thus be seen that the suitability of a colony for resting place formation varies with the amount of diagonal, vertical and peripheral branching that it shows.

A further indication of the evident ease with which coral skeleton can be modified was provided by a colony of *Acropora palmata* in which the corallites were thickened in an area brushed by the seemingly light touch of the expanded tentacles

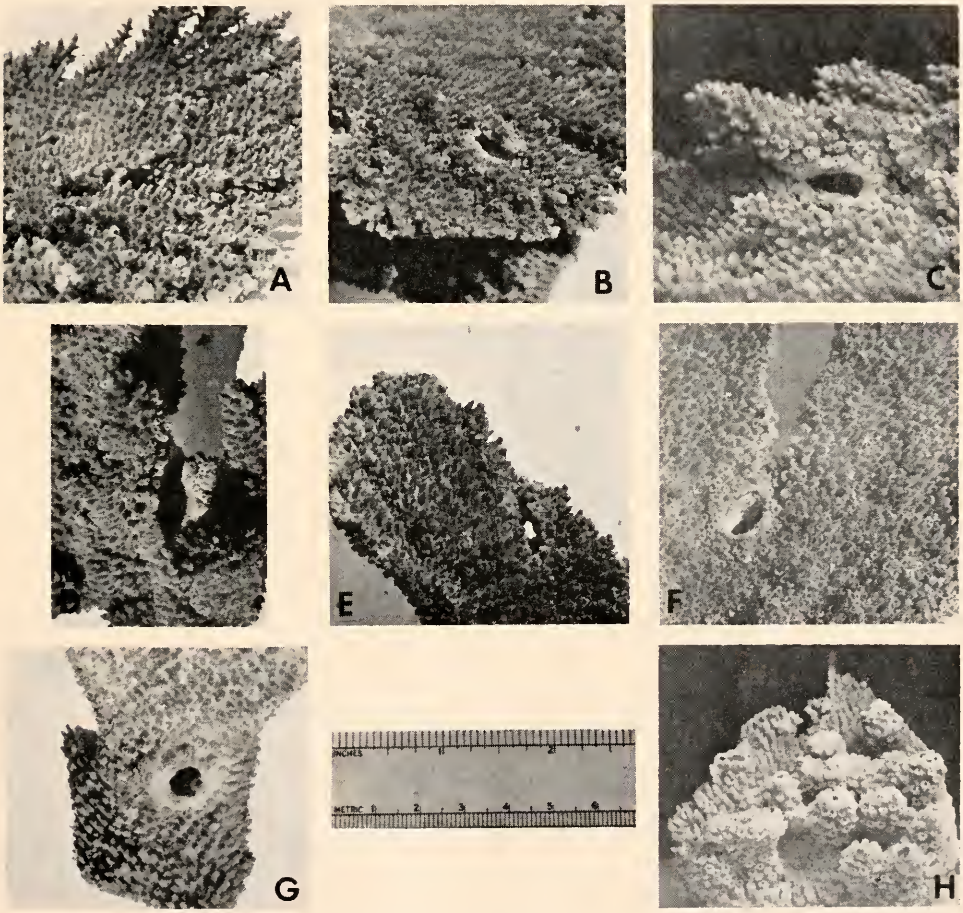

FIGURE 2. Modifications induced in *A. palmata* by *D. acanthophora*. A, Early stage of crevice formation in colony showing much peripheral branching. B, Older and better developed crevice in the same colony. C, Crevice under a diagonal branch. D, Well developed crevice at side of a colony. E, Crevice enclosed anteriorly. F, Pit. G, Pit. H, Modified space between vertical branches.

of an adjacent tube worm. The well known fact that many branching corals, including *A. palmata,* have different growth forms under different wave conditions (Wood-Jones, 1907; Vaughan, 1919; Storr, 1964) also shows that the deposition of coral skeleton can be much influenced by factors in the environment.

The above formation of resting places is not unique, as similar though more regular modifications are known to be induced in their coral hosts by the various gall-crabs of the family Hapalocarcinidae. The best known species, *Hapalocarcinus marsupialis* Stimpson has been well described by Potts (1915). Here the male is free-living while the female forms galls in branching corals of the family Pocilloporidae. The coral grows around the crab, eventually enclosing her except for several small openings in the top of the gall. Potts noticed a malformation of both corallites and polyps on the inside of the gall and regarded this and the growth of the gall itself as the reaction of the coral to the crab's respiratory currents. Although respiratory currents may play a part in resting place formation in *Acropora palmata* it seems more likely that the coral is responding to contact with the crab itself. This is particularly true in the case of crevices and the modified spaces between branches where the coral is altered in the region actually touched by the crab but not in the area in front of the crab against which the exhalant respiratory current must press.

The remaining members of the Hapalocarcinidae form pits and crevices in various massive corals (Fize and Seréne, 1957). The only previously reported case of decapod modifying *Acropora* is that of the uncommon Indo-Pacific gall shrimp, *Paratypton siebenrocki* Balss.

The commensal

Domecia acanthophora looks much like any free-living xanthid crab and as evidenced by the individuals on *A. cervicornis* and *A. prolifera* is not dependent on the existence of a resting place. This is in marked contrast to the gall-crabs and gall-shrimp mentioned above which are very much modified structurally and are seemingly unable to survive outside of their galls for any length of time. Furthermore, *D. acanthophora* is not an obligate commensal of a particular group of corals. Rathbun (1930) reported it "among sponges and branches of corals and in holes of dead corals and stones" and mentioned the corals *Meandrina* and *Porites*. Rathbun (1921) found it on *Acropora* at Barbados. At La Parguera, Dr. P. W. Glynn (personal communication) found this crab commonly on *Acropora palmata* and in beds of *Porites furcata* in shallow water on reef flats.

The specimens of *D. acanthophora* observed on *Acropora* in nature moved very little. When disturbed, however, they could move very rapidly across the coral colony. Those inhabiting *A. palmata* were very reluctant to leave their resting places and generally would not do so until touched with forceps. In the laboratory crabs showed a strong negative phototaxis and a low thigmokinesis. These traits are of course shared by many benthic organisms. If there is competition among crabs for resting places, some type of territorial behavior might be expected. This was never observed.

Many colonies of *Acropora* showed no evidence of commensals and so were not collected. Table I shows the results of several collections that were made.

Note the fairly close correspondence which exists between the number of resting places found on *A. palmata* and the number of crabs on the colony. This may be due to predation of unprotected crabs by the many small fish which hover around the coral or more likely to the tendency of the crab to keep moving until it is adequately sheltered. The small excess of crabs over resting places may be due to (a) crabs wandering over the colony, or (b) crabs inhabiting structurally unmodified shelters, or (c) two crabs inhabiting the same resting place. The first two alternatives were noticed occasionally, the third only once. The vast majority of resting places seen in nature were found to be inhabited.

Some differences were noticed in the crabs found on the two species of coral. Of those shown in Table I, the largest taken from *A. prolifera* was a female of 10.1 mm. carapace breadth while *A. palmata* contained 5 males and 13 females of this size or larger. As can be seen from Table I the sex ratio is about equal in crabs inhabiting *A. prolifera* while on *A. palmata* there were twice as many females as males.

TABLE I

Colonies and portions of colonies of Acropora collected from Enrique Reef between July 20 and August 5, 1965

		A. palmata													*A. prolifera*								
Colony number		1	2	3	4	5	6	7	8	9	10	11	12	13	1	2	3	4	5	6	7	8	9
crabs found	males		3	2	5		5	1				5	4	1	1	1	4	1		2		1	2
	ovigerous females	1	6	3	5	1	6		2			7	4	1	1	1			1		1	2	1
	non-ovigerous females	1	4		5		2	1		1		4	3	1	2		1		1	2			
	juveniles (under 5 mm.)				2		1		1	1		2	1		2					1		1	
	Total	2	13	5	17	1	14	2	3	2	0	18	12	3	6	2	5	1	2	5	1	4	3
Number of resting places found on colony		1	10	4	14	1	11	1	3	2	0	16	7	3	0	0	0	0	0	0	0	0	0

To investigate the mobility of *D. acanthophora,* the resting places on several adjacent colonies of *A. palmata* were mapped and examined on 8 of the next 13 days. After each day's observation the crabs inhabiting the resting places were destroyed. The results, shown in Table II, permit several observations to be made. (1) Crabs do not have to make their own resting places, but will readily inhabit those they find vacant. Thus once a resting place is formed, it could be used by many crabs during the life of the colony. (2) There is evidently considerable movement of *Domecia* on to the colony and if the originally vacated resting places had been left undisturbed, they would probably all have been re-occupied in about a week. (3) Much of the movement involved smaller individuals. It would have been highly desirable to measure each crab, but unfortunately they were so agile or else so well entrenched they could not be caught without destroying them. (4) Some resting places are strongly preferred.

Feeding habits

Specimens of *D. acanthophora* kept in the laboratory would hardly move unless disturbed and refused to eat fragments of shrimp or fish. The main activities that were observed were flicking movements of the first antennae and periodic extensions and withdrawals of the second maxillipeds.

Most brachyuran crabs are carnivores or scavenging omnivores and their stomachs contain fragments of more or less identifiable animal or plant material. The stomachs of 14 *D. acanthophora* contained no animal organisms, no fragments of muscle, chitin or other tissue and only a very occasional strand of algae. The bulk of the material in the stomachs was a white, structureless mass containing variable amounts of large bacteria. Small mineral grains and sponge spicules were also commonly found. One crab contained many small yellowish cells about 10 microns in diameter which may have been zooxanthellae.

TABLE II

Re-inhabitation of various resting places on adjacent areas of A. palamata. All crabs were destroyed following each day's observations

		Day								
		1	2	3	4	7	8	9	11	14
Crevices	a	P	—	—	—	—	—	—	—	—
	b	P	—	L	M	M	—	M	S	M, S
	c	P	—	—	—	—	—	S	—	—
	d	P	P	—	M	—	—	—	—	—
	e	P	—	—	—	S	—	—	—	—
Pits	A	P	P	S	S	—	—	S	S	—
	B	P	P	—	—	S	—	—	—	—
	C	P	P	S	—	—	—	—	—	—
	D	P	P	S	S	—	—	S	—	—
Spaces between vertical branches	1	—	—	—	—	—	—	—	—	—
	2	P	P	—	—	—	—	—	—	—
	3	P	—	P	—	M	—	S	—	—
	4	—	—	—	—	—	—	—	—	—

S, M, L = small, medium or large sized crabs
P = crab present, size not noted
— = no crab present

The mouthparts of two fairly typical non-commensal crabs, the shore crab, *Carcinus maenas* and a spider crab, *Hyas coarctatus,* are very similar even though the crabs belong to different superfamilies (Borradaile, 1922; Hartnoll, 1963). When the mouthparts of *D. acanthophora* (Fig. 3) are compared with those of the above crabs, however, a number of marked differences can be seen. (1) The dactyl of the third maxillipeds of *Domecia* has a much longer tuft of terminal hairs. (2) The armature of the dactyl of the second maxillipeds is very different. In *D. acanthophora* the dactyl is considerably broadened and bears many stout setae on its inner surface. These are arranged in rows with each row being composed of setae of a different length. The longest ones are the most ventral, above which are rows of progressively shorter and more dorsal setae. On either side of the terminal portion of each seta there is a row of up to 12 lateral bristles. Each seta ends in a peculiar tripartite paddle which turns upwards at a right angle. This paddle is composed of the tip of the seta and a thickened bristle on either side. The longer setae curve dorsally and have a greater number of lateral bristles than do the shorter ones. (3) The first maxillipeds and second maxillae have better developed setae on their medial surfaces. (4) The mandibles are more weakly calcified while the mandibular palp has a different shape and fewer setae.

The teeth of the gastric mill also differ from those found in typical brachyurans. In ten species of spider crab, Hartnoll (1963) found that the lateral teeth of the

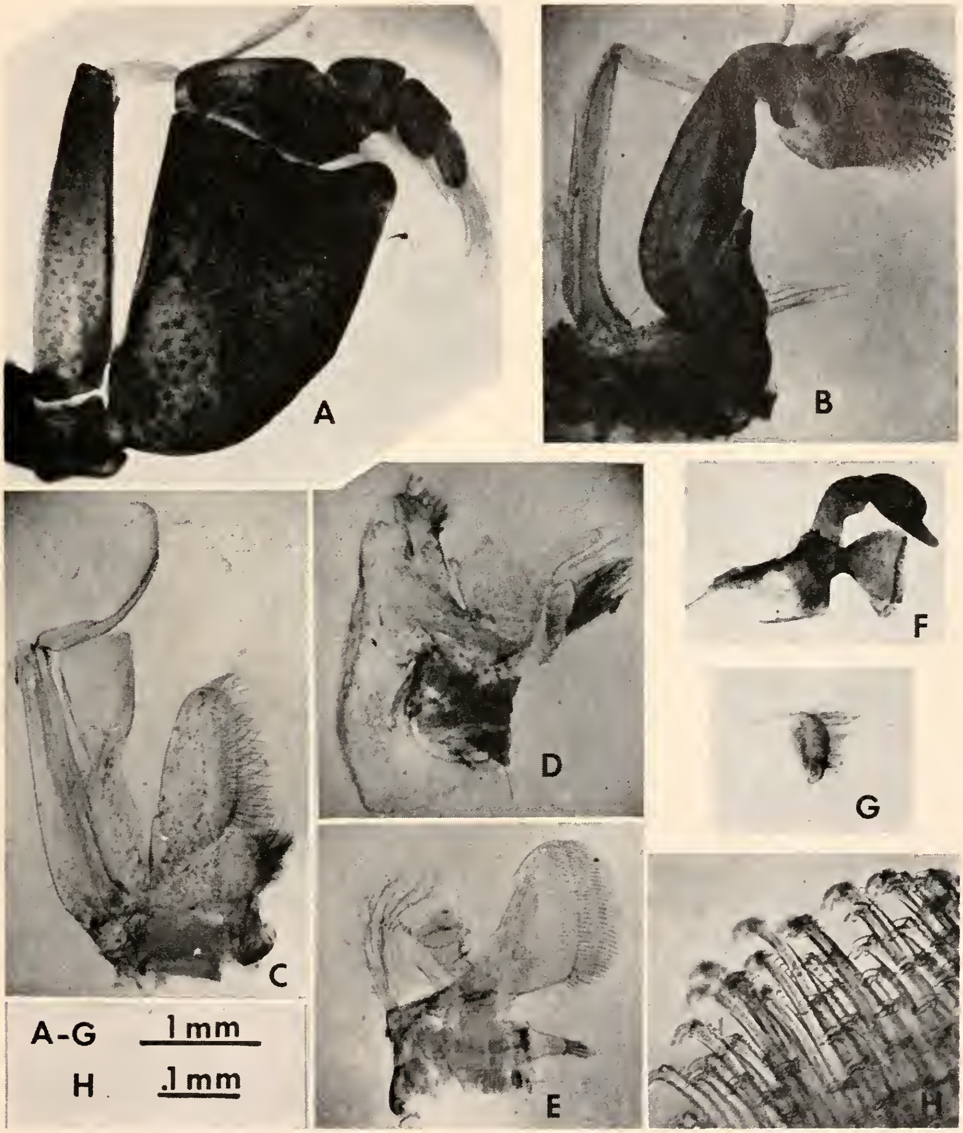

FIGURE 3. A–G, Ventral view of right mouthparts of female *Domecia acanthophora*, 12.4 mm. carapace breadth. A, Third maxilliped. B, Second maxilliped. C, First maxilliped. D, Second maxilla. E, First maxilla. F, Mandible. G, Paragnath. H, Dorsal view of spines on distal end of the dactyl of the second maxilliped.

zygocardiac ossicles had from 3–7 cusps and from 0–7 ridges. In *Carcinus* they have 5 fairly blunt cusps and 8 ridges (Potts, 1915). In *D. acanthophora,* however, the lateral teeth consist of 13–16 fairly slender cusps whose points vary from rounded to sharp and about 20 ridges.

In the light of the above observations it is probable that *D. acanthophora* removes its food from the water with the second maxillipeds. It is not a typical filter-feeder, however. The only possible filtering structure is the dactyl of the second maxillipeds and this is of relatively small size and lacks the abundant pinnate setae usually found in the filter of filter-feeding crustaceans (Marshall and Orr, 1960). The dactyls of ten crabs were carefully examined and although the crabs had not been preserved until an hour after being caught, in nine cases the setae contained amorphous material with inclusions of sand grains. In two cases, the setae were largely covered with a sheet of this material while in the others small pieces were entangled on some of the paddles. There were a very few small particles on the setae which did not seem to be in a matrix of other material. This material is very similar to that found in the stomach and is doubtless scraped off the dactyl by the setae on the medial surfaces of the first maxillipeds and second and first maxillae, and placed between the protruding mandibles.

A likely source of this amorphous material is the organic detritus drifting in the water since descriptions of it agree closely with the stomach contents. Hunt (1925) describes detritus from Plymouth as a pale brown flocculus containing living micro-organisms, fragmented skeletal parts and sand grains. Riley (1963) states that (p. 273) "organic aggregates in Long Island Sound commonly consist of pale yellowish or brownish amorphous matrices with inclusions of bacteria, silt particles and sometimes phytoplankton." Coral mucus is also a possible source of the material eaten by *D. acanthophora.* It is shed abundantly by *Acropora* and could contain sand grains and spicule fragments which settle on it. I doubt if it is a major food, however. Crabs were never observed to eat mucus and do not seem to move around the colony as they might if they were collecting it. One crab was seen to remove a strand of mucus that had drifted into its mouthparts.

ZOOGEOGRAPHY

The genus *Domecia* occurs in tropical coral habitats around the world and contains three species. The best known is *D. hispida* Eydoux and Souleyet. It extends from the Red Sea across the Pacific to the west coast of America and is apparently restricted to colonies of *Pocillopora.* The stomach contents of a specimen of *D. hispida* from the Galapagos were identical to that reported above for *D. acanthophora.* The mouthparts of this specimen were very similar to those of *D. acanthophora* and the two species doubtless have the same feeding habits. Although *Hapalocarcinus* forms galls in *Pocillopora,* deformation of this coral by *D. hispida* has not been reported.

The second species, *D. glabra* Alcock, extends across the Indo-Pacific from Madagascar to Tahiti and has been taken from *Acropora* (Garth, 1964; Patton, 1966). Resting place formation has not been reported but may occur on the plate-forming species.

As to the third species, Guinot (1964) has shown that specimens of *Domecia* from the tropical Atlantic do not represent *D. hispida* as previously supposed but constitute a distinct, though very similar species, *D. acanthophora* (Desbonne and Schramm). Furthermore, the American specimens *D. acanthophora* forma *acanthophora*, which occur from South Carolina to Brazil, can be distinguished from African specimens *D. acanthophora* forma *africana* Guinot. Little is known of the habitat of the African form except that it is found among corals.

The genus *Acropora* is very well developed in the Indo-Pacific and has quite a varied fauna of commensal decapods (Garth, 1964; Patton, 1966). The types with fairly close branches have the most commensals but even species similar to those of the Caribbean have more than one commensal species. The question then arises as to why *Domecia acanthophora* is the only decapod commensal with Puerto Rican *Acropora*. The answer may well be an historical one. The present-day hermatypic Atlantic corals are believed to result from a time when the Tethys Sea connected the Atlantic with the Indian Ocean. This connection was permanently broken in the Miocene. Later in the Tertiary the families Acroporidae and Pocilloporidae had an enormous development in the Indo-Pacific (Wells, 1956), producing numerous species, many of which offer a great deal of shelter to commensals. It seems likely that the development of the present extensive commensal faunas of these two families would have followed or paralleled this expansion of their hosts. If this was so, the *Acropora* fauna which evolved in the Indo-Pacific would have had no opportunity to reach the Caribbean. There is no record of *Acropora* occurring in the East Pacific (Durham and Allison, 1960) and the only possible tropical connection between the Indo-Pacific and the Atlantic would have had to involve the East Pacific and a Central American waterway.

Pocillopora, on the other hand, though absent in the Caribbean since the Miocene (Durham and Allison, 1960), is common in the East Pacific and contains several of the typical Indo-Pacific *Pocillopora* commensals including abundant *Domecia hispida* (Crane, 1947; Garth, 1948). The last Central American seaway was closed either in the late Miocene (Durham and Allison, 1960) or the Pliocene (Lloyd, 1963). Although the first record of *Pocillopora* in the East Pacific is in the Pleistocene (Durham and Allison, 1960), it may have been there earlier along with its commensals and prior to the closing of connections with the Atlantic. If this was the case, then *D. hispida* could have crossed over and established itself on Atlantic *Acropora*. The ability of *Domecia* to feed on detritus could have been sufficient reason for it being the only one of the *Pocillopora* commensals to successfully cross into the Atlantic and transfer to a new host. Following the separation of the two oceans, the Atlantic *Domecia* would have evolved into a separate species.

An alternative explanation for the presence of *Domecia* in the Atlantic is that the genus is older than the other commensal decapods and evolved before the severing of the Tethys connection. This seems less likely since the great morphological similarity of *D. hispida* and *D. acanthophora* suggests that they have a relatively recent common ancestry.

SUMMARY

1. The xanthid crab *Domecia acanthophora* was collected from three species of the coral *Acropora* at Enrique Reef, La Parguera, Puerto Rico.

2. The commensal was most commonly found on the flattened coral sheets of *Acropora palmata*. Here the majority of crabs inhabit various types of structural deformation of the coral which are called resting places. These are believed to be formed by the growth of the coral around and to some extent away from a resting crab.

3. Although undisturbed crabs remain quite motionless, ones which are disturbed are capable of rapid movement over the colony. There is at least some movement of crabs around the reef as vacated resting places will be re-occupied by new crabs.

4. The mouthparts of *D. acanthophora* differ from those found in typical crabs. In particular, the mandible is weakly calcified and the second maxilliped possesses rows of peculiar paddle-tipped spines on the distal margin of the dactyl. The most likely food for the animal seems to be organic detritus which it separates from the surrounding water.

5. The genus *Acropora* harbors numerous commensal decapods in the Indo-Pacific but apparently only one in the Caribbean.

LITERATURE CITED

Almy, C. C., Jr., and C. Carrión-Torres, 1963. Shallow-water stony corals of Puerto Rico. *Caribb. J. Sci.*, **3**: 133–162.

Borradaile, L. A., 1922. On the mouth-parts of the shore crab. *J. Linn. Soc. London*, **35**: 115–142.

Crane, J., 1947. Intertidal brachygnathous crabs from the west coast of tropical America with special reference to ecology. *Zoologica*, **32**: 69–95.

Durham, J. W., and E. C. Allison, 1960. The geologic history of Baja California and its marine faunas. *Systematic Zool.*, **9**: 47–91.

Fize, A., and R. Seréne, 1957. Les Hapalocarcinidés du Viet-Nam. *Arch. Mus. Hist. Nat. Paris*, (7) **5**: 3–202.

Garth, J. S., 1948. The Brachyura of the "Askoy" Expedition with remarks on carcinological collecting in the Panama Bight. *Bull. Amer. Mus. Nat. Hist. 92* art. 1, pp. 1–66.

Garth, J. S., 1964. The Crustacea Decapoda (Brachyura and Anomura) of Eniwetok Atoll, Marshall Islands, with special reference to the obligate commensals of branching corals. *Micronesica*, **1**: 137–144.

Glynn, P. W., 1963. *Hermodice carunculata* and *Mithraculus sculptus*, two hermatypic coral predators. Assoc. Island Marine Lab. Caribb. 4th Meeting. Curacao; pp. 16–17.

Guinot, D., 1964. Les trois espèces du genre *Domecia* (Decapoda, Brachyura): *D. hispida* Eydoux & Souleyet, *D. glabra* Alcock, et *D. acanthophora* (Desbonne & Schramm). *Crustaceana*, **7**: 267–283.

Hartnoll, R. G., 1963. The biology of Manx spider crabs. *Proc. Zool. Soc. London*, **141**: 423–496.

Hunt, O. D., 1925. The food of the bottom fauna of the Plymouth fishing grounds. *J. Mar. Biol. Assoc.*, **13**: 560–599.

Lloyd, J. J., 1963. Tectonic history of the South Central-American orogen. *Amer. Assoc. Petrol Geol. Mem.*, **2**: 88–100.

Marsden, J. R., 1962. A coral-eating polychaete. *Nature*, **193**: 598.

Marshall, S. M., and A. P. Orr, 1960. Feeding and nutrition. *In:* T. H. Waterman (ed.), The Physiology of Crustacea, vol. 1: 227–258. Academic Press, New York.

Patton, W. K., 1966. Decapod Crustacea commensal with Queensland branching corals. *Crustaceana*, **10**: 271–295.

Potts, F. A., 1915. *Hapalocarcinus*, the gall-forming crab with some notes on the related genus *Cryptochirus*. *Papers Dept. Marine Biology Carnegie Institution*, **8**: 33–69.

RATHBUN, M. J., 1921. Report on the Brachyura collected by the Barbados-Antigua Expedition from the University of Iowa in 1918. *University of Iowa Stud. Nat. Hist.*, **9**: 65–90.

RATHBUN, M. J., 1930. The cancroid crabs of America of the families Euryalidae, Portunidae, Atelecyclidae, Cancridae and Xanthidae. *Bull. U. S. Nat. Mus.*, **152**: 1–609.

RILEY, G. A., 1963. Organic aggregates in seawater and the dynamics of their formation and utilization. *Limnol. Oceanog.*, **8**: 372–381.

STORR, J. F., 1964. Ecology and oceanography of the coral-reef tract, Abaco Island, Bahamas. Spec. Pap. Geol. Soc. America, No. 79, 98 pp.

VAUGHAN, T. W., 1915. The geologic significance of the growth-rate of Floridian and Bahaman shoal-water corals. *J. Wash. Acad. Sci.*, **5**: 591–600.

VAUGHAN, T. W., 1919. Corals and the formation of coral reefs. *Smithsonian Institute Ann. Rep.*, **1917**: 189–238.

WELLS, J. W., 1956. Scleractinia. *In:* R. C. Moore (ed.), Treatise on invertebrate paleontology. Part (F) Coelenterata: F328–F444. Geol. Soc. Amer. & Univ. of Kansas Press.

WOODS-JONES, F., 1907. On the growth forms and supposed species in corals. *Proc. Zool. Soc. London*, **1907**: 518–556.

AMOUNT, LOCATION, PRIMING CAPACITY, CIRCULARITY AND OTHER PROPERTIES OF CYTOPLASMIC DNA IN SEA URCHIN EGGS[1]

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The presence of large amounts of DNA in the cytoplasm of the mature egg of many species of animals has been reported by a number of early workers (for summaries see Brachet, 1962; Haggis, 1964; Grant, 1965; Monroy, 1965; Tyler and Tyler, 1966b). Values several hundred times that of the nucleus have been reported in eggs of sea urchins and frogs which have been the most extensively investigated material. However, the methods employed in the early determinations did not clearly distinguish between DNA and materials, such as polysaccharides and RNAs, that might interfere with the determinations, and, in fact, as the methods have become more refined the reported values have dropped. Thus, in *Paracentrotus lividus* Hoff-Jørgensen (1954) obtained about 20 times the haploid (H) value by microbiological assay and Whiteley and Baltzer (1958) obtained values at the 32-cell stage by a fluorometric method that extrapolate to a similar value for the unfertilized egg. In *Hemicentrotus lividus* Sugino *et al.* (1960) reported about 37 × H on the basis of thymidine determinations. Pikó and Tyler (1965) obtained approximately 13 × H and 8 × H, respectively, in *Lytechinus pictus* and *Strongylocentrotus purpuratus* by differential and buoyant density centrifugation methods. Eberhard and Mazia (1965), from fluorometric measurements, estimated about 180 × H in *S. purpuratus* but indicated that the material that reacted with the 3,5-diaminobenzoic acid dihydrochloride in their experiments might not all be DNA. Baltus *et al.* (1965), using a microfluorometric method, and Bibring *et al.* (1965), using centrifugation methods, found about 25 × H in *Arbacia lixula.*

The location of the egg cytoplasmic DNA has also been uncertain. A few years ago substantial evidence first appeared for the presence of DNA in the mitochondria of cells of a number of organisms, including chick embryo (Chèvremont, 1962; Nass and Nass, 1963), mammalian tissues (Swift *et al.*, 1964; Schatz *et al.*, 1964b), protozoa (Steinert *et al.*, 1958; Rudzinska *et al.*, 1964), molds (Luck and Reich, 1964), yeast (Schatz *et al.*, 1964a), ferns (Bell and Mühlethaler, 1964), maize (Ris, 1962). The evidence has accumulated since these first investigations and DNA is now generally considered to be an integral part of the mitochondrion (for reviews and further evidence see Gibor and Granick, 1964; Swift, 1965; Nass

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[1] Supported by grants from the National Science Foundation (GB-28) and from the National Institutes of Health (GM 12777) and (CA 08014). The authors wish to acknowledge the effective technical assistance of Peter N. Redington, Edward E. Vivanco and Robert Watson.

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[3] Contribution No. 3475 from the Division of Chemistry and Chemical Engineering.

et al., 1965; Rabinowitz *et al.*, 1965; Suyama and Preer, 1965; Dawid, 1966; Corneo *et al.*, 1966; Sinclair and Stevens, 1966). It seemed possible, then, that the egg cytoplasmic DNA might be entirely contained in these bodies. In fact a calculation (Pikó and Tyler, 1965) based upon an estimate of the volume occupied by mitochondria (*ca.* 9%) in sea urchin eggs (Shaver, 1956, 1957) and reported values (*e.g.*, Schatz *et al.*, 1964a) of DNA in mitochondria, gives approximately the amount of cytoplasmic DNA that has been found.

Baltus and Brachet (1962) (*cf.* Roller, 1963; Brachet, 1965) found about two-thirds, at least, of the cytoplasmic DNA of frog's eggs to be associated with large particles (pigment granules and yolk platelets) that sediment at low speed. Yolk spherules of certain types, however, have been shown to be derived from mitochondria at least in some species (Lanzavecchia, 1960, 1965; Ward, 1962; *cf.* Srivastava, 1965). In eggs of the clawed toad *Xenopus laevis*, and the frog *Rana pipiens*, Dawid (1965, 1966) has found DNA in the mitochondria. The total obtained in the mitochondrial preparations accounts for some 65 to 80% of the cytoplasmic DNA.

Comparisons have been made of cytoplasmic with nuclear DNA with respect particularly to buoyant density and estimated molecular weight. Bibring *et al.* (1965) indicate that in *P. lividus* it has a high molecular weight and a base composition similar to that of nuclear DNA. They also report that the buoyant density of the DNA (presumably mostly cytoplasmic) extracted from eggs is similar to that found in sperm. Carden *et al.* (1965) also found in *Arbacia punctulata* that nuclear and cytoplasmic DNAs exhibit the same buoyant density in CsCl gradients. In the experiments reported here the cytoplasmic DNA is found to differ in buoyant density from that of the nucleus.

The sedimentation behavior of this material indicated a similarity to the DNAs of various viruses that are known to have a closed circular structure of uniform circumference, as shown electron microscopically by Weil and Vinograd (1963) for the DNA of the polyoma virus and by Kleinschmidt *et al.* (1963) and Chandler *et al.* (1964) for the replicating form of bacteriophage ØX174. Also, as Vinograd *et al.* (1965) have shown, the circles are composed of double helices that are in a superhelical form unless scissions are introduced into one or the other of the two strands. Circular DNAs with circumferences ranging from 0.5 to 9.7 microns have been found by electron microscopy in DNA preparations from boar sperm by Hotta and Bassel (1965). Recent studies by Borst and Ruttenberg (1966) and by Van Bruggen *et al.* (1966) have shown the presence of circular DNA in mitochondrial preparations from chick and mouse liver and from beef heart. The circles were of uniform circumference (*ca.* 5.45 microns). Sedimentation velocity analyses revealed two components with standard sedimentation coefficients of 39–42S and 27–29S that correspond to the twisted and relaxed circular forms described by Vinograd *et al.* (1965). Similar findings have been reported by Sinclair and Stevens (1966) for mouse liver mitochondria. Our own studies indicate that the DNA of the mitochondria of sea urchin eggs is also of the circular type as will be reported here and in more detail in a subsequent paper.

Evidence that cytoplasmic DNA may be potentially active in oocytes or mature eggs has been provided by experiments of Shmerling (1965) on sturgeon oocytes, showing that DNA extracts that must contain predominantly cytoplasmic DNA possessed priming activity for DNA and for RNA synthesis equal to that of DNA extracted from the sperm. There is also evidence for *in vivo* activity of the cytoplasmic DNA of eggs. Thus Mezger-Freed (1963) has reported that artificially activated enucleated frog eggs (with the nucleus in an attached exovate) synthesize DNA about as rapidly as do the fertilized eggs during early cleavage. Similarly in sea urchins Baltus *et al.* (1965) report synthesis of DNA by artificially activated non-nucleate fragments and, in addition, the synthesis of RNA. In the present experiments the DNA extracted from the mitochondria of sea urchin eggs was found to be capable of serving as primer for RNA synthesis.

Materials and Methods [4]

(A) *Egg and sperm samples*

The sea urchins *Lytechinus pictus* and *Strongylocentrotus purpuratus* were used in these experiments. The eggs were obtained by KCl injection and handled in artificial sea water as described elsewhere (Tyler and Tyler, 1966a). After removal of the gelatinous coat in pH 5 sea water and thorough washing the eggs were suspended in 0.55 *M* KCl following three approximately 30:1 (v/v) washings in this solution. Samples were removed from the penultimate suspension for counting (Tyler and Tyler, 1966a).

"Dry" sperm (semen) was collected as it exuded freely from dissected gonads and diluted with artificial sea water to a stock solution of 0.5 to 1.0%. Spermatozoal counts were made with the Coulter electronic counter having a 30 μ orifice. As a check, counts were also made by hemocytometer.

(B) *Preparation of homogenates*

The preparative procedure was similar in principle to that of Kay (1964); namely, the use of detergent followed by salt extraction. In addition EDTA was included further to reduce the possibility of nuclease activity and to serve as a buffer supplemental to the egg material itself. In these experiments one volume of packed KCl-washed eggs was mixed with three volumes of the homogenization medium [4% sodium dodecyl sulphate (SDS), 0.08 *M* ethylenediamine tetraacetate and 9% ethanol, pH 7.8] and stirred gently for 20 to 30 minutes at 20° C., with a Teflon rod. CsCl (optical grade, Harshaw Chemical Company) was then added, with continued slow stirring for about one-half hour, to give the desired final densities. These were 1.5 gm./cm.^{3} in the initial experiments in which attempts were made by differential centrifugation to remove Dische-interfering materials and 1.70 gm./cm.^{3} in the later experiments in which the DNA was isolated by buoyant density centrifugation. Marker C^{14}-DNA (see below) dissolved in 3.75 molal CsCl, when used, was added at this time in the ratio of 1 volume to 150 or 300 volumes of homogenate. In some cases the homogenates were stored at

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[4] *Abbreviations used in the text:* DNA, deoxyribonucleic acid; RNA, ribonucleic acid; DNase, pancreatic deoxyribonuclease; SDS, sodium dodecyl sulphate; EDTA, ethylenediamine tetraacetate; TCA, trichloroacetic acid; SSC, standard saline-citrate (0.15 *M* NaCl, 0.015 *M* Na citrate, pH 7); ATP, GTP, UTP, CTP, adenosine-, guanosine-, uridine-, and cytidine-triphosphate, respectively.

– 20° C. before the addition of CsCl, and this had no apparent effect on the amount of DNA later obtained or on its properties.

(C) *Marker DNA*

Fertilized *Lytechinus pictus* eggs, at a density of 40,000 eggs/ml., were raised until the gastrula stage in artificial sea water containing 50 μC./ml. C^{14}-thymidine (30 μC./μM specific activity, New England Nuclear Corp.). After several washings with ice-cold sea water and 0.55 *M* KCl, the embryos were homogenized with SDS-EDTA and the DNA was isolated by buoyant density centrifugation in CsCl solution. Fractions of 0.15 ml. were collected by puncturing near the bottom of the centrifuge tubes and examined for absorption at 260 mμ and for radioactivity. A single sharp peak of absorption, and of radioactivity, was obtained. The fractions containing this material were pooled and stored in a freezer at – 20° C. In two separate preparations made in this manner one had a specific activity of 12×10^{-6} mC./mg. DNA and the other 16×10^{-6}. In the experiments involving the use of marker DNA the amount of radioactivity was measured in a Packard Tri-Carb scintillation counter at 50% counting efficiency by a method described elsewhere (Tyler, 1966).

(D) *DNA determinations*

Measurements of the DNA content of the various preparations in the initial experiments were made by the diphenylamine reaction of Dische (1930). This was used on hot acid extracts (15 minutes extraction with an equal volume of 10% TCA in a boiling water bath) of spermatozoal suspensions, and egg homogenates and the various fractions thereof. Aliquots, mixed with Dische's reagent and blank, respectively, were heated in a boiling water bath for 10 minutes, cooled rapidly and allowed to stand at room temperature for one hour. The absorptions were read with a Beckman DU spectrophotometer and DNA values calculated by comparison with a deoxyadenosine standard. In some cases, spectral absorption curves between 500 and 700 mμ were taken with a Cary Model 15 spectrophotometer.

For the purified materials isolated by the centrifugation methods the DNA values were determined directly from measurements of the absorption at 260 mμ, assuming a value (Ogur and Rosen, 1950) of 0.050 mg./O.D. unit.

(E) *Centrifugation*

All high speed centrifugations were done in a Spinco model L or L2-65 ultracentrifuge with an SW-39 or SW-65 rotor. The conditions of centrifugation are indicated in the individual experiments. The preformed density gradients employed in these experiments were prepared by use of a gradient-making device of the type described by Leif and Vinograd (1964) but capable of filling three tubes at one time.

The results of the buoyant density experiments were evaluated by the procedures described by Vinograd and Hearst (1962). All buoyant densities were calculated by the marker method. A value of 1.710 gm./cm.^{3} for *E. coli* DNA was assumed.

(F) *Isolation of mitochondria*

Following the experience of Shaver (1956) with the sea urchins used in these experiments, mitochondrial preparations were made by homogenization of KCl-washed eggs in 3 volumes of a solution containing 0.3 *M* sucrose, 0.36 *M* KCl, 0.03 *M* Tris-HCl and 0.003 *M* EDTA at pH 7.6. Homogenization was done by hand with a loose fitting Teflon pestle in a Potter-Elvehjem tube of about 2 cm. diameter containing 15 ml. of suspension, for a total of 10 strokes in the cold at relatively slow speed. The homogenates were first centrifuged for 10 minutes at 1200 to 1500 rpm (SW-25 rotor of the Spinco model L centrifuge, 4° C.) to remove nuclei and large egg fragments. The supernatant was then centrifuged at 12,000 rpm in the same rotor for 20 minutes. The resulting pellet was resuspended in homogenization medium and recentrifuged under the same conditions. Further processing is described below.

(G) *Priming activity*

The ability of the extracted DNA to serve as primer for RNA synthesis as first described by Weiss and Gladstone (1959), was measured from the incorporation of C^{14}-labeled CTP in a system containing also the other three trinucleotides (GTP, ATP, UTP), and an RNA polymerase prepared from *E. coli* by the method of Chamberlain and Berg (1962). We are indebted to Dr. K. Marushige for these materials.

Results and Comments

(A) *DNA content*

(1) *Determination by Dische reaction*

When the "DNA" of homogenates of whole eggs of *S. purpuratus* and *L. pictus* was directly assayed by the Dische reaction, the amount per cell ranged from 90 to 150 times that of the sperm. These are similar to the values reported by a number of earlier investigators as noted above. However, as Elson *et al.* (1954) noted with sea urchin eggs, the color that is obtained is not that characteristic of deoxyribose alone. In our tests the reaction mixture showed an absorption peak at 530 mμ. Figure 1 illustrates an absorption curve for the egg material (curve A) along with that of the sperm (D) and one for deoxyadenosine (F).

One initial attempt to remove the interfering material was done by differential centrifugation of the homogenates brought to a density of 1.5 gm./cm.^{3} with CsCl. After 14 hours of centrifugation at 35,000 rpm (Spinco SW-39 rotor) the material separated into a small gelatinous bottom pellet, a rubbery top layer (occupying about 5% of the tube when the final homogenate contains some 12 to 14% of eggs) and a clear intermediate fluid. Dische reactions were run on the combined pellet and intermediate fluid and on the top layer in the two species. Determinations were also made on sperm. The top layer contained about four-fifths of the 595 mμ absorption values of whole egg homogenates but the absorption curve is similarly abnormal (curve B of Figure 1). The possibility that "trapped" DNA may be contained in it is considered in a later section. For the combined pellet and clear

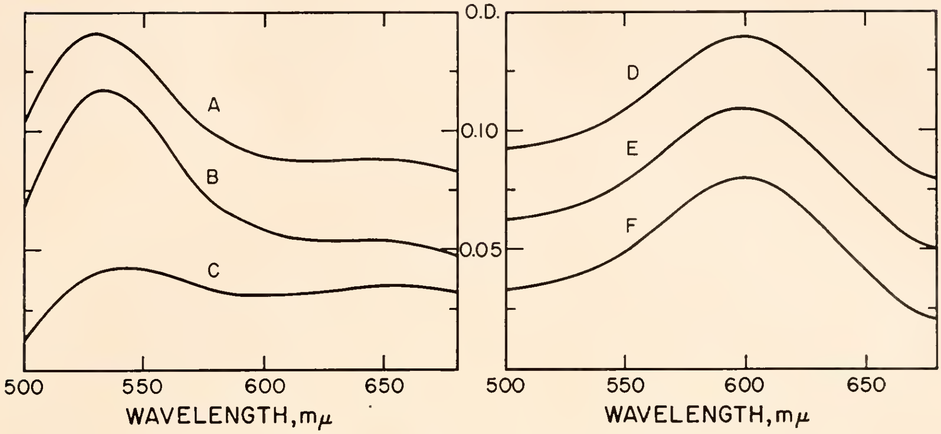

FIGURE 1. Spectral absorption curves of colors obtained with the Dische reaction. (A) Whole egg homogenate of *S. purpuratus;* (B) top layer and (C) combined pellet and clear layer obtained after differential centrifugation of homogenates of *S. purpuratus* eggs (see text); (D) sperm of *L. pictus;* (E) pre-purified and alpha amylase-treated extract from eggs of *L. pictus;* (F) deoxyadenosine standard.

layer the following values (in micrograms "DNA" per million eggs) were obtained:

S. purpuratus	17.7, 19.3, 20.5, 22.0; av. 19.9
L. pictus	19.6, 16.2, 16.4; av. 17.4

For the sperm the values were as follows:

S. purpuratus	0.72, 0.82; av. 0.77
L. pictus	0.84, 0.90; av. 0.87

The spectrum of the Dische-reacted combined pellet and clear layer (curve C of Figure 1) is somewhat less abnormal than that of the other preparations.

After exploration of a number of methods of disposing of the interfering material the use of alpha amylase proved effective. Preparations that are obtained by a single buoyant density separation show a turbid polysaccharide layer (see section 3) at the same level of the tube where the DNA is located. When this fraction, after it is precipitated with alcohol and redissolved, is treated with alpha amylase, as described in section 3, it gives a typical deoxyribose spectrum in the Dische reaction, as illustrated in curve E of Figure 1. By this procedure the DNA values obtained for the two species were:

L. pictus	8.0 pg./egg
S. purpuratus	3.6 pg./egg

These are similar to the values obtained by UV absorption measurements on purified DNA as described below.

(2) *Evidence against "trapping" of DNA in the top layer*

In order to examine the possibility that DNA might be trapped in the top layer that forms upon centrifugation of homogenates in the CsCl solutions, use was made of the C^{14}-labeled gastrula DNA. In five experiments with *L. pictus* a sample of the labeled DNA was added to the homogenate and determinations made of the distribution between the top layer and the clear fluid after prolonged centrifugation in the CsCl solutions employed for buoyant density separations (see below). The values obtained in these experiments for the ratio of labeled DNA in the top layer to that in the clear fluid ranged from 1:99 to 2:98.

(3) *Direct determinations on purified material*

Preparations of DNA were made from whole eggs of *S. purpuratus* (3 experiments) and *L. pictus* (5 experiments) by buoyant density centrifugation of homogenates adjusted to a density of 1.70 gm./cm.^{3} with CsCl and containing radioactive (marker) gastrula DNA. Centrifugation was for at least 50 hours at 35,000 rpm at 10–12° C. in the SW-39 rotor of the Spinco model L centrifuge. A small amount of solid CsCl is present at the bottom of the tube at the end of the run. For collecting the fractions, then, the hypodermic needle is introduced above this layer (approximately 7 mm. from the bottom). Usually 15 to 20 fractions were collected and the radioactivity of small aliquots determined. The fractions comprising and surrounding those with the radioactive DNA were pooled and CsCl solution (1.70 gm./cm.^{3}; in 0.02 *M* Tris–HCl pH 7.6) added so as to give a volume sufficient to fill the centrifuge tubes, which were then re-run as before.

It was noted, early in these experiments, that a band of visible turbidity, later identified as polysaccharide (Pikó and Tyler, 1965; Segovia *et al.,* 1965), appeared in the region of the centrifuge tube where the marker DNA was located. This material continued to appear at the level of the DNA upon repeated centrifugations and it obscured the O.D.$_{260}$ readings. For removal of this material two methods were explored. One was centrifugation of alcohol-precipitated and redissolved fractions on preformed CsCl density gradients (1.22 to 1.65 gm./cm.^{3} for 4 hours at 35,000 rpm, 20° C.) in which the polysaccharide sediments (*ca.* 100S) well ahead of most of the DNA. The other method was simply to incubate a solution (0.5 *M* KCl, 0.01 *M* Tris, 0.005 *M* EDTA, pH 7) of the alcohol-precipitated DNA and polysaccharide-containing fractions with α-amylase (Worthington, 2 × crystallized, at 0.75 mg./ml. for 1 hour at 37° C.). This method proved to be the more effective. The digestion with alpha amylase was generally done with the fractions collected after the first or second centrifugation. Following this the buoyant density centrifugations and collection of the fractions were repeated two times.

After the final buoyant density centrifugation, absorbances of the fractions at 260 and 280 mμ were read on a Beckman DU spectrophotometer. Radioactivity (of the marker DNA) determinations on aliquots again served to locate the DNA-containing region, and to provide an additional basis for quantitation. The fractions collected after the final centrifugation by this procedure showed a single O.D.$_{260}$ peak at a level corresponding to a density near 1.70 gm./cm.^{3} The marker DNA showed a single peak of radioactivity in the region of 1.69. This is illustrated in Figures 2a and 2b. In one of these experiments an aliquot was treated with DNase (Worthington, electrophoretically purified, 0.1 mg./ ml. at 37° C. for 30 minutes) before the final buoyant density centrifugation. This resulted in complete elimination of both the O.D.$_{260}$, and the radioactivity, peaks.

DNA prepared in this way shows a typical absorption spectrum, as illustrated in Figure 3. In 5 experiments with unfertilized eggs of *L. pictus* and 3 experiments with *S. purpuratus*, in which the determinations were made by this method, the following values were obtained for the content of DNA in micrograms per million eggs.

L. pictus	7.9, 9.3, 7.5, 8.2, 8.4; av. 8.26 ± 0.30
S. purpuratus	3.5, 2.8, 3.6; av. 3.30 ± 0.25

(4) *Extraction of DNA from eggs labeled during oogenesis*

Further evidence for the effectiveness of the extraction procedure has been obtained in an experiment in which the DNA was labeled radioactively during oogenesis by the general procedure described by Tyler and Tyler (1966a). In this experiment a female *L. pictus* received two successive injections, intracoelomically, of 200 microcuries of H^3-thymidine (6 curies/mM) at a one-month interval and the eggs were collected one month after the second injection. About 100,000 eggs were obtained, and tests on an aliquot showed about two-thirds of the radioactivity to be in acid-precipitable form. Upon extraction by the procedures employed here (see section 3) all the labeled material, that was identified as DNA, was found, upon buoyant density centrifugation, to be in a layer at a density near 1.70 gm./cm.[3] This material contained about 0.1% of the originally injected radioactivity. In addition there was an approximately equal amount of radioactivity at the top of the tube. This material, upon treatment with preincubated (1 hour) pronase (Calbiochem, final concentration 2 mg./ml. in 0.25 *M* CsCl, 0.005 *M* Tris, 0.001 *M* EDTA, 5% ethanol, pH 7.6; incubated at 50° C. for 12 hours) lost more than 90% of its originally acid-precipitable radioactivity. It

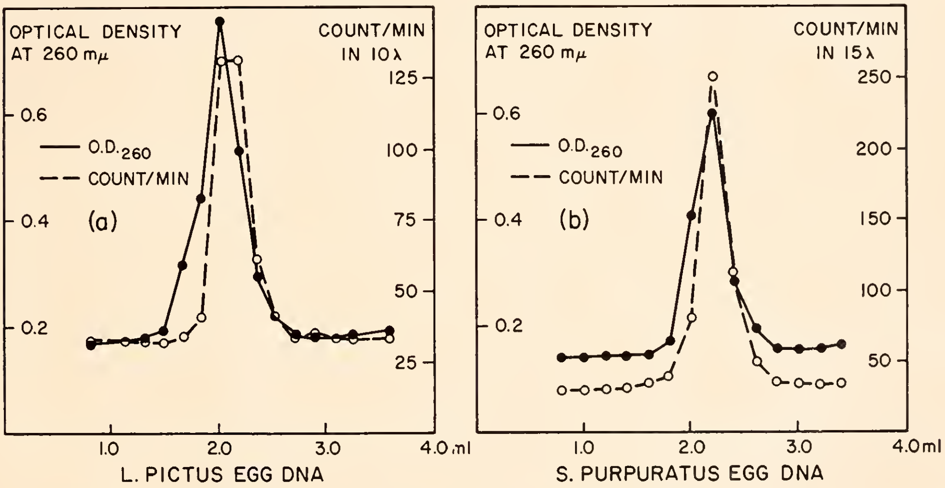

FIGURE 2. Cesium chloride gradients of DNA from unfertilized eggs of *L. pictus* and *S. purpuratus*. The C^{14}-labeled nuclear marker DNA (from *L. pictus* gastrulae) bands at a somewhat lower density than the bulk of the cytoplasmic DNA (for procedures see text).

may, then, be concluded that the extraction procedure yields practically all the DNA obtainable from the egg.

(B) *Presence of DNA in mitochondria and yolk*

Two sets of experiments were run in which homogenates of *L. pictus* eggs (of determined number) were subjected to differential centrifugation, as described under Methods, so as to separate a 250 × *g* nuclear (N) pellet, a 18,000 × *g* mitochondria + yolk (M + Y) pellet and a supernatant (S) fraction. Two or three consecutive buoyant density centrifugations in CsCl solution were performed on each fraction, as described in section A3, the treatment with alpha amylase being applied on the fractions collected after the first centrifugation. From O.D.$_{260}$ readings the following amounts of DNA in micrograms per million eggs were obtained. The distribution in per cent of the total is given in parentheses.

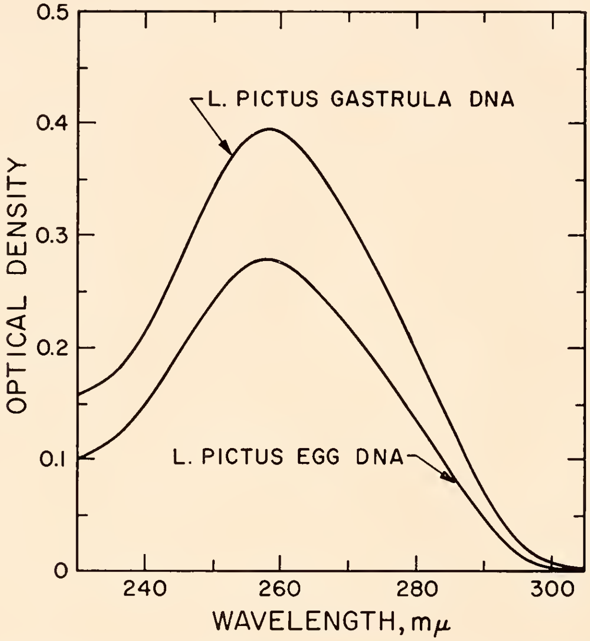

FIGURE 3. Ultraviolet absorption spectra of purified DNA in 0.015 *M* NaCl–0.0015 *M* sodium citrate, pH 7, from gastrulae and unfertilized eggs of *L. pictus*.

Experiment 1: N = 1.51 (20.5%); M + Y = 5.03 (68%); S = 0.85 (11.5%)
Experiment 2: N = 0.51 (8.5%); M + Y = 3.84 (65%); S = 1.57 (26.5%)

In experiment 1 no separate determination of DNA content of whole eggs was made, but if the average value of 8.26 pg. per egg from other experiments is taken then the recovery here is about 90%. In experiment 2 parallel determination of DNA content was made on an aliquot of the initial whole egg homogenate. This gave a value of 8.40 micrograms per 10^6 eggs, as corrected for 76% recovery of marker DNA. If we assume a similar recovery for the above fractions then the corrected total for them is 7.8 micrograms, which would indicate very little, if any, loss in the fractionation procedure.

As the results show, the bulk of the DNA is found in the M + Y fraction. The DNA content of the N fraction is lower in experiment 2 than in experiment 1. In experiment 1 this fraction had not been washed. Microscopic examination has shown that some mitochondria and yolk spherules do sediment with this fraction. This probably accounts, then, for the value of its DNA content being almost twice that expected for the nuclei alone.

The S fraction is free of microscopically visible mitochondria and yolk particles, and shows in the two experiments 11.5 and 26.5%, respectively, of the total DNA content extracted from the eggs. Whether or not this DNA may be derived from damaged mitochondria or yolk cannot be stated at present.

An aliquot of the M + Y fraction in the second experiment, with *L. pictus*, was subjected to further processing by centrifugation on preformed linear gradients of sucrose solutions (from 0.93 *M* to 1.88 *M* sucrose in 0.003 *M* Tris, 0.0025 *M* EDTA, pH 7.6) in the SW-25 rotor of the Spinco model L at 25,000 rpm for 2 hours at 4° C., 2.5 ml. of the suspension of M + Y fraction being layered on 25 ml. of gradient in each tube. Under these conditions the yolk (Y) remains on the top of the gradient while the mitochondria (M) sediment as a band that is visible as a cloudy layer some 4 mm. wide at a region of the tube where, as determined by subsequent weighing, the density is 1.18 gm./cm.^{3} This fraction and the top one were collected, diluted with three volumes of 0.5 *M* KCl (containing 0.05 *M* Tris, 0.005 *M* EDTA, pH 7.6) and centrifuged at 12,000 rpm (SW-25 rotor) for 20 minutes. The pellets were suspended in SDS-EDTA solution, CsCl added to a density of 1.70 gm./cm.^{3} and the solutions subjected to two buoyant density centrifugations and fractionations, with intervening alpha amylase digestion, as described previously. The following values were obtained for DNA in micrograms per million eggs.

Experiment 2: M = 2.07; Y = 0.72

The sum represents 73% of the amount of DNA present in the M + Y fraction, as listed above.

In a separate experiment (3) an M + Y fraction of *L. pictus* eggs was prepared and all of this used for preparation of M and Y fractions as described in this section. The following values for DNA content (micrograms per million eggs originally extracted) were obtained.

Experiment 3: M = 2.47; Y = 0.35

From these experiments it is clear that the bulk of the DNA appears in the mitochondrial fraction. In the two experiments (2 and 3) in which M + Y was separated into M and Y the ratios (M:Y) of DNA content were 3:1 and 7:1, respectively. From the sedimentation behavior, including the wide separation of the two fractions, and from microscopic examination it is unlikely that the yolk fraction contains any significant amount of mitochondria as such. Considering also the lack of any appreciable trapping of marker DNA in that layer it is most reasonable to conclude that the DNA found therein is a component of the yolk spherules. The differences in the relative amounts of DNA obtained from the mitochondrial and yolk fractions in the two experiments may be explained by the sensitivity of these particles to damage during the extraction procedures. The results of cesium chloride buoyant density centrifugation of DNA from mitochondria and from yolk are illustrated in Figure 4a, 4b. Both DNAs behave similarly, forming bands at somewhat higher density than the added radioactively labeled nuclear DNA.

In a preparation made by Dr. E. R. Berger, now of the Veterans Administration Hospital, Sepulveda, approximately 2140 mitochondria and 2280 yolk spherules were counted on a montage of electron micrographs of a thin section (maximum diameter) of an egg of *L. pictus*. From these figures, and values of 2.0 microns for the diameter of a yolk spherule and equivalent spherical diameter of 0.8 micron for a mitochondrion, it may be estimated that there are some 80,000 yolk spherules and some 200,000 mitochondria per egg. This corresponds to the approximately 3:1 ratio for the DNA found in mitochondria and yolk in experiment 2.

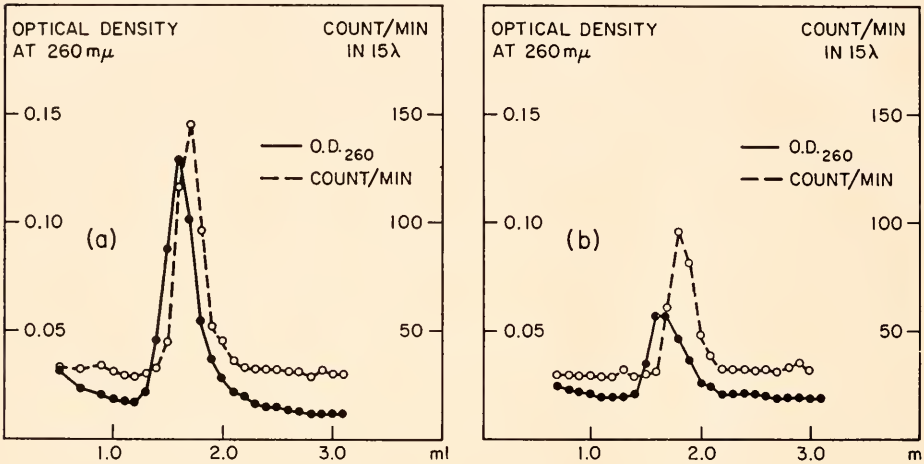

FIGURE 4. Cesium chloride gradients of DNA from (a) isolated mitochondria and (b) yolk of *L. pictus*. Each preparation contained added C^{14}-labeled marker DNA from *L. pictus* gastrulae. The band of radioactivity is at a lower density than that of $O.D._{260}$ absorption for both the mitochondrial and yolk-DNA.

(C) *Priming activity for RNA synthesis*

Purified DNA from eggs of *L. pictus* and *S. purpuratus* was tested for ability to serve as primer for RNA synthesis. DNA that is preponderantly (more than 99%) nuclear was prepared from late blastulae and from plutei of *S. purpuratus,* to serve as a basis for comparison. The measurements were made of the incorporation of radioactive label into RNA (material precipitable by 10% trichloroacetic acid) in a system containing, in 0.25 ml., the following: 0.1 μmole C^{14}-cytosine triphosphate (1.4 μC./μ*M*), 10 μmoles Tris buffer, pH 8, 1 μmole $MgCl_2$, 0.25 μmole $MnCl_2$, 3 μmoles beta mercaptoethanol, 0.1 μmole each of ATP, GTP and

UTP, and the RNA polymerase from *E. coli.* The following values were obtained in terms of counts per minute (cpm at 30% counting efficiency) per 5 μg. DNA above a background of about 90 cpm for the complete mixture minus the DNA.

S. purpuratus:	Unfertilized egg DNA (80% cytoplasmic)	= 4,056 cpm
	Blastula DNA (< 1% cytoplasmic)	= 3,618 cpm
	Pluteus DNA (< 1% cytoplasmic)	= 4,305 cpm
L. pictus:	Unfertilized egg DNA (90% cytoplasmic)	= 3,027 cpm

These initial values are all in the same general range. If only the nuclear DNA of the egg preparations were active, the values for these preparations would have been very much lower (one-fifth to one-tenth of those obtained). It may be concluded, then, that the cytoplasmic DNA can function as primer for RNA synthesis.

(D) *Some physical properties of the cytoplasmic (mitochondrial) DNA*

Detailed studies of various physical properties of the cytoplasmic DNA of *L. pictus* are in progress and will be reported elsewhere. Here some preliminary information is given concerning its density, melting behavior, sedimentation properties and microscopic appearance.

(1) *Buoyant density.* In the preparative buoyant density centrifugations in CsCl solution of whole egg homogenates, with radioactive marker DNA included, the O.D.$_{260}$ readings consistently show a peak at a higher density (*ca.* 1.70 gm./cm.^{3}) than the peak of radioactivity of the marker (*ca.* 1.69 gm./cm.^{3}). This is illustrated in Figures 2a, 2b. This is also true for the DNA obtained from isolated mitochondria and yolk as shown in Figures 4a, 4b. In further buoyant density centrifugations of purified whole egg DNA of *L. pictus* in the analytical (Beckman Spinco Model E) centrifuge three bands were observed in scans at 265 mμ. The buoyant densities were 1.693, 1.703 and 1.719 gm./cm.^{3} (see Fig. 5). The relative amounts of DNA in these three bands were of the order of 1:7:1. Scans at 280 mμ again revealed three bands in which the ratios of the areas were approximately the same as at 265 mμ. In the same rotor sperm DNA and gastrula DNA form single bands at 1.693 gm./cm.^{3} The 1.703 band evidently represents the bulk of the cytoplasmic DNA. The nature of the 1.719 band is not, as yet, known.

(2) *Melting temperature.* Determinations of melting profiles were made on purified (as described in section A3) DNA preparations from spermatozoa, late gastrulae and unfertilized eggs of *L. pictus.* Sedimentation analysis showed that the latter preparation contained a negligible fraction of intact, *i.e.,* covalently closed, circular DNA. After dialysis and storage in one-tenth strength standard saline citrate (SSC = 0.15 *M* NaCl; 0.015 Na citrate; pH 7.0), the measurements were made in SSC. From the profiles (see Figure 6) the average melting temperatures (Tm) are 84.0° C. for sperm and gastrula DNA and 86.8° C. for the unfertilized egg DNA. In the latter case the value is not entirely attributable to the cytoplasmic DNA since there is some 10% each of nuclear DNA and an unidentified component of a buoyant density in CsCl of 1.719. This may explain the atypical shape of the melting curve of the unfertilized egg DNA (*cf.* also Vinograd and Lebowitz, 1966). From the Tm for the whole egg DNA a guanine-

cytosine (G-C) content of 42% is calculated (Marmur and Doty, 1962; Schildkraut and Lifson, 1965), which approximates the G-C content of 44% calculated (Schildkraut *et al.*, 1962) from the CsCl buoyant density of 1.703 gm./cm.^{3} of the major peak (presumably mitochondrial DNA) obtained from unfertilized eggs. For sperm DNA and gastrula DNA the Tm and the buoyant density indicate a G-C content of 35 and 34%, respectively.

(3) *Sedimentation behavior.* In four separate experiments in which preparations (see section A3) of DNA from whole eggs of *L. pictus* were centrifuged on preformed linear CsCl gradients (1.30 to 1.40 gm./cm.^{3} in the SW-65 rotor for 2 hours at 50,000 rpm, 20° C.) the nuclear DNA sedimented to the bottom of the tube and the cytoplasmic DNA sedimented in two distinct bands, corresponding to sedimentation coefficients of about 23S and 28S, respectively. Approximately equal amounts of the two fractions were obtained, the amounts varying in different preparations. As noted in the introduction, the presence of two such components is indicative of the two forms of circular DNA (twisted and open circles) described by Vinograd *et al.* (1965) and found in mitochondria by van Bruggen *et al.* (1966). Further studies on this material will be reported in a separate paper.

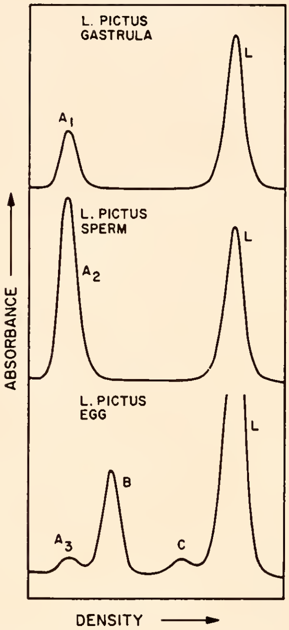

FIGURE 5. Tracings of direct scans at 265 mμ of buoyant density bands (in CsCl) of three preparations of DNA of *L. pictus* after 25 hours of centrifugation in the same rotor at 44,770 rpm in the Beckman model E centrifuge. L: density marker DNA (1.731 gm./cm.^{3}) of *Micrococcus lysodeikticus.* A_1, A_2 and A_3: DNAs identified as nuclear in the three preparations and with similar buoyant densities of 1.693. B: DNA identified as derived from mitochondria and yolk and with a buoyant density of 1.703. C: Unidentified nucleic acid band of buoyant density 1.719.

(4) *Electron microscopic observations.* Purified DNAs from whole eggs, from mitochondrial fractions and from gastrulae of *L. pictus* were prepared for electron microscopy according to the method of Kleinschmidt *et al.* (1965). For this purpose a small amount (0.2 ml.) of a solution of ammonium acetate (1.5 *M*; pH 7) containing DNA at about 4 micrograms per ml. and cytochrome *c* at 0.1 mg. per ml. was allowed to flow down an inclined glass slide onto a solution of 0.1 *M* ammonium acetate in a large dish. Electron microscope grids coated with formvar were touched to the surface of the solution, passed through 95% ethanol, 0.0001 *M* uranyl acetate solution in 0.001 *M* HCl and isopentane. The preparations were examined in a Philips EM200 and micrographs taken at a film magnification of 5000 ×. (We are indebted to Mr. James Wetmur of the Division of Chemistry for the use of uranyl acetate in this procedure.)

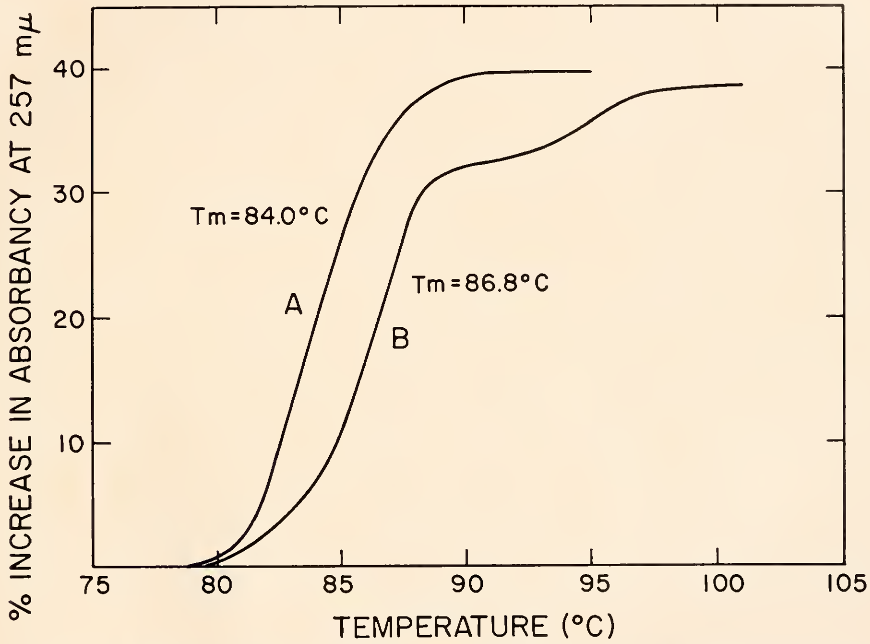

FIGURE 6. Meeting profiles of DNAs (in 0.15 *M* NaCl–0.015 *M* sodium citrate, pH 7) from *L. pictus*. Curve A: DNA from sperm and from gastrulae. Curve B: DNA from whole unfertilized eggs.

In the preparations from both whole eggs and mitochondrial fractions the DNA was seen to be present mostly in the form of closed circular filaments, whereas none of these were seen in the preparations from gastrulae. The latter is estimated to contain less than 1% of cytoplasmic DNA. Examples of the circular DNA are shown in Figure 7. Both twisted and open circles are seen. A considerable uniformity of size of circles was observed. Measurements of 144 perimeters gave values ranging from 3.75 to 4.83 microns with a mean of 4.45 and a standard deviation of 0.25. We are indebted to Mr. Donald Blair of the Division of Chemistry for providing us with the foregoing quantitative results.

DISCUSSION

(A) *DNA content*

The present determinations of DNA content of the unfertilized eggs have given values in the general range of those obtained by the more recent workers on sea urchin eggs. However, the values are significantly lower than any previously reported except for that of Marshak and Marshak (1953). By an isotope dilution method they obtained a value of 10 × H (haploid) for *Arbacia punctulata* but attributed most of this to contamination with somatic cells and polar bodies and concluded there was some 3 × to 4 × H of cytoplasmic DNA. They also concluded, from the failure to obtain a Feulgen reaction, that the nucleus of the unfertilized egg lacked DNA. However, measurements by Hinegardner (1961) on isolated nuclei of *E. mathaei* and of *S. purpuratus* showed that these contain 1 × H of DNA and others (*e.g.*, Burgos, 1955) have obtained a positive Feulgen reaction. In the present experiments, the areas under the buoyant density bands in the analytical ultracentrifuge indicate the nuclear DNA to be present in approximately the haploid amount in *L. pictus* eggs.

In the present work evidence has been presented that the material on which the final determinations were made was, in fact, DNA. Also, the monitoring with radioactively labeled DNA permitted an assessment to be made of the effectiveness of recovery during the preparative procedures. This was reinforced by the results of the experiment in which DNA was extracted from eggs in which it had been labeled during oogenesis. It seems reasonable to conclude, then, that the present values of 8.26 pg. and 3.30 pg. per egg for *L. pictus* and *S. purpuratus*, respectively, are close to the actual content of macromolecular DNA in these cells. With regard to other species of echinoids that have been examined, since none of these have an egg size larger than that of *L. pictus* and since the nuclear DNA is closely the same for various species (*cf.* Tyler and Tyler, 1966a), it seems reasonable to expect that the total DNA should be in the same range as the values reported here. The much higher values that have been reported, in the absence of substantial evidence of specificity of the analyses, would seem then to be attributable at least in part to the presence of interfering materials.

In the two species that have been used in the present work the difference in DNA content correlates with differences in egg size. Correlation with egg size may account for the much greater values that have been reported for amphibian eggs in investigations in which attempts have been made to eliminate interfering materials. Thus, Baltus and Brachet (1962) report 0.069 μg. for the axolotl, Haggis (1964) reports 27,000 × H for *Rana pipiens*, and Dawid (1965) gives values 600 to 1000 × H for *R. pipiens* and *Xenopus laevis*.

(B) *Presence in mitochondria and yolk*

The present results show, as suggested earlier (Pikó and Tyler, 1965), that the bulk of the cytoplasmic DNA is present in the mitochondria. This accords with the current findings on amphibian (*R. pipiens* and *X. laevis*) eggs by Dawid (1966) who reports that at least two-thirds of the DNA is associated with the mitochondrial particles. As noted in the introduction the general occurrence of DNA in mitochondria is now well established from investigations with various organisms throughout the animal and plant kingdoms.

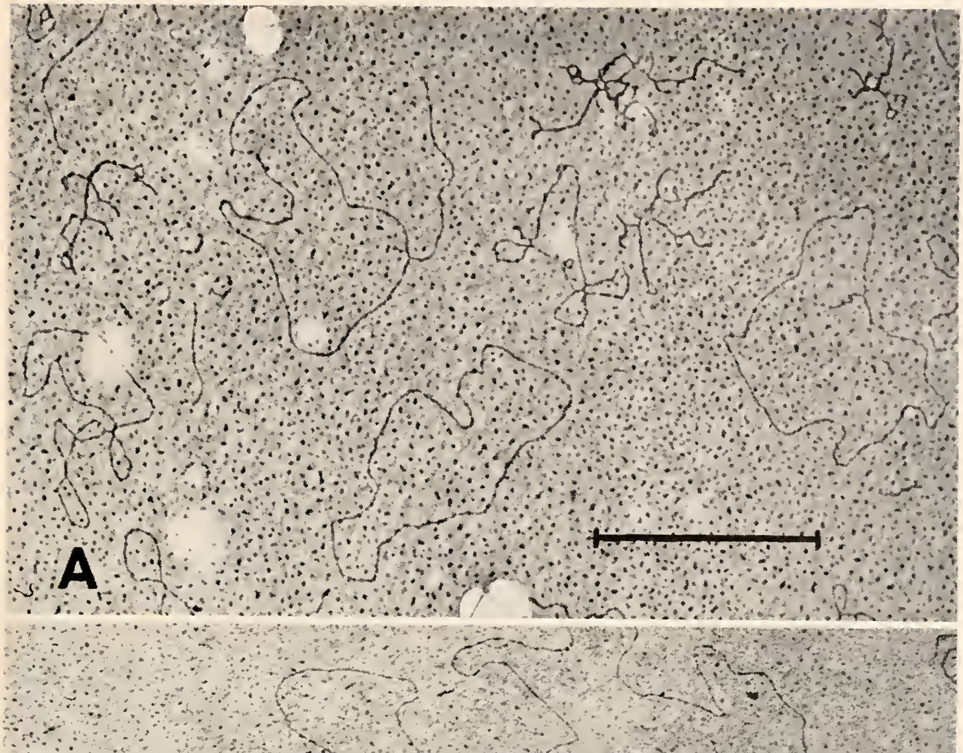

FIGURE 7. Electron micrographs of DNA from mitochondria (A) and of DNA from gastrulae (B) of *L. pictus*. The line represents one micron.

Our results also show the presence of DNA in the preparations of yolk spherules. Although there is a possibility that this DNA is simply adherent to the yolk spherules this seems unlikely in view of the preparative procedures. The evidence accords then with that of Baltus and Brachet (1962), who found about two-thirds of the DNA (as detected by a fluorometric method) of eggs of *Pleurodeles* to be associated with the particles (mostly yolk) sedimentable at low speeds ($280 \times g$). It also accords with the evidence of Brachet and Ficq (1964, 1965), obtained by use of radioactively labeled actinomycin, that DNA is an integral component of the yolk spherules.

There is, in addition, convincing evidence, from studies on frogs (Lanzavecchia, 1960, 1965; Ward, 1962) that at least some of the yolk spherules, or possibly all, are derived from mitochondria. This seems likely to be the case for eggs of animals in general (*cf.* Srivastava, 1965). If one assumes no increase in amount of DNA upon transformation of a mitochondrion into a yolk spherule, then our finding of a much lower content of DNA in the total mass of yolk than in the mass of mitochondria is readily understandable on the basis of the relative volumes of the two particles. Thus in *Lytechinus,* the unfertilized egg contains, by our rough estimate, about 200,000 mitochondria, and 80,000 yolk spherules. From the areas under the bands in the analytical buoyant density centrifugations it appears that seven-ninths of the total DNA, namely 6.4 μg. per 10^6 eggs, is in these particles. This gives 2.3×10^{-17} grams per particle, or 1.4×10^7 daltons.

(C) *Metabolic properties*

The present results showing priming activity for RNA synthesis on the part of the sea urchin cytoplasmic DNA add to the evidence (see introduction) for such activity on the part of the cytoplasmic DNA of eggs of other species (*cf.* Shmerling, 1965 for fish; Baltus *et al.,* 1965 for sea urchins; and Dawid, 1965 for Amphibia). That sea urchin egg mitochondria are capable also of protein synthesis was shown earlier by Nakano and Monroy (1958) and Giudice (1960). It appears, then, that in mitochondria of sea urchin eggs the complete systems of RNA-dependent protein synthesis and DNA-dependent RNA synthesis are present, as has been demonstrated for mitochondria of other origin (*cf.* Kroon, 1963; Kalf, 1964).

Detailed measurements are not as yet available concerning the extent to which the mitochondrial activity accounts for the protein synthesis that occurs upon fertilization in sea urchins, but according to Nakano and Monroy (1958) and Giudice and Monroy (1958) this remains insignificant during the first three or four hours of development. The evidence for potential activity of mitochondrial DNA does not then alter the conclusions that have been drawn earlier from experiments with non-nucleate fragments (Tyler, 1963, 1965; Denny and Tyler, 1964; Brachet, Ficq and Tencer, 1963) and with actinomycin D (Gross and Cousineau, 1963, 1964) of the existence in the unfertilized egg of an inactive ("masked") messenger RNA that becomes active upon fertilization. In fact the experiments with actinomycin D provide particularly strong arguments against the possibility that an activation of mitochondria might be responsible for the great increase in protein synthesis that occurs upon fertilization, inasmuch as it is known (Kalf, 1964) that incorporation of amino acid into protein by intact mitochondria is sensitive to actinomycin. Further arguments are provided by the fact, demonstrated originally by Hultin (1961), that the difference between unfertilized and fertilized eggs is exhibited also by cell-free systems which, from the method of preparation, are evidently free of mitochondria.

(D) *Physical properties*

The buoyant density centrifugation in CsCl solutions in the preparative ultracentrifuge consistently showed the cytoplasmic DNA to have a higher density than the nuclear, in the range of 1.70 to 1.71 gm./cm.^{3} A determination by the analytical ultracentrifuge gives a value of 1.693 for the nuclear and 1.703 for the bulk of the cytoplasmic DNA in *L. pictus* eggs, and a small (10% of the total DNA) band at 1.719. While the latter is a nucleic acid band, as indicated by the O.D.$_{260}$–O.D.$_{280}$ ratios and other properties, it could be a DNA–RNA hybrid rather than DNA alone. Detailed studies of physical properties of the various components are in progress and will be reported elsewhere.

For the nuclear and cytoplasmic DNA of other species there have been reports both of similarities and of differences in buoyant density. Thus in *Arbacia punctulata*, Carden *et al.* (1965) reported similar buoyant densities for the two DNAs. In *Rana pipiens,* Dawid (1965, 1966) reports that the two DNAs have the same buoyant density (1.702 gm./cm.^{3}) while in *Xenopus laevis* the cytoplasmic DNA is slightly denser (by 0.002 gm./cm.^{3}) than the nuclear. In chickens 1.707 gm./cm.^{3} for the mitochondrial DNA, and 1.698 gm./cm.^{3} for the nuclear, are reported by Rabinowitz *et al.* (1965) and Borst and Ruttenberg (1966). The latter, and also Sinclair and Stevens (1966) report similarity of buoyant density for mitochondrial and nuclear DNAs of various mouse tissues, while Schneider and Kuff (1965) report a somewhat lower buoyant density (1.699 gm./cm.^{3}) for mitochondrial DNA from rat liver as compared with nuclear DNA (1.703 gm./cm.^{3}). In different species of animals, then, the two DNAs may be alike or different in density. The presently available data indicate that even related species may differ in this regard. Even within the same species differences may occur in the buoyant density of mitochondrial DNA, as Mounolou *et al.* (1966) have shown for "petite" mutants of yeast.

Our determinations of melting temperature showed the cytoplasmic DNA to have a Tm of 86.8° C. as compared with 84.0° C. for the nuclear DNA. As noted in the results, this corresponds to a guanine-cytosine content of 42% as compared with 35% for the nuclear DNA. This is in good agreement with the values (44 and 34%, respectively) calculated from the buoyant densities.

The sedimentation experiments revealed two main components in the preparations of cytoplasmic DNA. As noted in the introduction, according to the analysis of Vinograd *et al.* (1965), this, along with other properties, indicated that the sea urchin cytoplasmic DNA might be in the form of circles which could, also, be of twisted and relaxed forms sedimenting at different rates. Examination by electron microscopy has corroborated the circular form. Further studies of this material are in progress. The data reported here show the circles to be of rather uniform size with perimeter close to 4.45 microns. This is near the values reported for mitochondrial DNA of chick and mouse liver and beef heart by van Bruggen *et al.*

(1966) and of mouse and rat liver (and several other tissues) by Sinclair and Stevens (1966).

On the basis of the present evidence from our material, and that of others, it would appear that all of the cytoplasmic DNA may be in circular form, and that filaments may represent breakage due to preparative procedures. In fact the relaxed circles are considered (*cf.* Vinograd *et al.*, 1965) to result from the occurrence of one or more single-strand scissions in the native material and, as preparative procedures improve, the filaments and extended circles become less frequent, as the twisted circles increase proportionately.

On the basis of the present figures it can be estimated that there are only one or two circles (of 4.45 microns perimeter) per mitochondrion of eggs of *L. pictus*. Whether the circular units are genetically alike in all mitochondria is one of the many interesting questions now open for investigation.

Addendum: While this paper was in press, an article appeared by M. M. K. Nass (1966) who reports that there are 2 to 6 circular DNA molecules per mitochondrion in mouse fibroblasts (L cells).

SUMMARY

1. Values of 8.26 ± 0.30 pg. (9.5 × haploid amount) for *Lytechinus pictus* and 3.30 ± 0.25 pg. (4.3 × haploid amount) for *Strongylocentrotus purpuratus* have been obtained for the DNA content per egg of these sea urchins. The methods involved repeated CsCl-buoyant density centrifugations, digestion of interfering polysaccharide, and monitoring of the procedures with added radioactively labeled DNA. The final determinations were made on material characterized by several criteria as highly purified.

2. Mitochondrial (M) and yolk (Y) fractions of differentially centrifuged homogenates of *L. pictus* eggs contain the bulk of the cytoplasmic DNA. It is uncertain to what extent the smaller variable amount (11.5 to 26.5%) found in the supernatant may be derived from breakdown of M- and Y-particles.

3. For distribution between M- and Y-fractions the best present value is considered to be about 3:1. Since yolk spherules are approximately one-third as numerous as mitochondria, the amount of DNA is estimated to be the same per particle, namely, 2.3×10^{-17} grams.

4. Evidence is presented that the cytoplasmic DNA of eggs of *L. pictus* and *S. purpuratus* can serve as primer in a DNA-dependent RNA-synthesizing system with approximately the same activity as nuclear DNA.

5. The cytoplasmic DNA of *L. pictus* eggs shows a buoyant density of 1.703 gm./cm.^{3} ac compared with 1.693 for the nuclear. A third nucleic acid band, equal in amount to the nuclear, has been found at a density of 1.719. The amount of nuclear DNA corresponds to the sperm (haploid) value.

6. Melting temperatures in standard saline-citrate are 84.0° C. for sperm and gastrula DNA and 86.8° C. for whole-egg DNA, indicating a guanine-cytosine content calculated from these values as 35% and 42%, respectively. These are similar to the values (34% and 44%) calculated from the buoyant densities.

7. Electron microscopic observations of DNA prepared from mitochondria of *L. pictus* show almost exclusively circles that measure about 4.45 microns in cir-

cumference. It is estimated that there are one or two such circular filaments of double-stranded DNA per mitochondrion or yolk particle.

8. Centrifugation of egg DNA of *L. pictus* in preformed CsCl gradients has revealed two main components with sedimentation coefficients of *ca.* 23S and 28S, indicative of the two forms of circular DNA.

LITERATURE CITED

Baltus, E., and J. Brachet, 1962. Le dosage de l'acide désoxyribonucléique dans les oeufs de Batraciens. *Biochim. Biophys. Acta,* **61**: 157–163.

Baltus, E., J. Quertier, A. Ficq and J. Brachet, 1965. Biochemical studies of nucleate and anucleate fragments isolated from sea urchin eggs. A comparison between fertilization and parthenogenetic activation. *Biochim. Biophys. Acta,* **95**: 408–417.

Bell, P. R., and K. Mühlethaler, 1964. Evidence for the presence of deoxyribonucleic acid in the organelles of the egg cells of *Pteridium aquilinum. J. Mol. Biol.,* **8**: 853–862.

Bibring, T., J. Brachet, F. S. Gaeta and F. Graziosi, 1965. Some physical properties of cytoplasmic deoxyribonucleic acid in unfertilized egges of *Arbacia lixula. Biochim. Biophys. Acta,* **108**: 644–651.

Borst, P., and G. J. C. M. Ruttenberg, 1966. Renaturation of mitochondrial DNA. *Biochim. Biophys. Acta,* **114**: 645–647.

Brachet, J., 1962. Nucleic acids in development. *J. Cell. Comp. Physiol.,* **60** (Suppl. 1): 1–18.

Brachet, J., 1965. Le rôle des acides nucléiques dans la morphogenèse. *Ann. Biol.,* **4**: 21–48.

Brachet, J., and A. Ficq, 1964. Détection cytochimique, au moyen d'actinomycine radioactive, de l'acide désoxyribonucléique (DNA) cytoplasmique des oeufs de Batraciens. *C. R. Acad., Sci. Paris,* **258**: 6258–6260.

Brachet, J., and A. Ficq, 1965. Binding sites of ^{14}C-actinomycin in amphibian oocytes and an autoradiography technique for the detection of cytoplasmic DNA. *Exp. Cell Res.,* **38**: 153–159.

Brachet, J., A. Ficq and R. Tencer, 1963. Amino acid incorporation into proteins of nucleate and anucleate fragment of sea urchin eggs: effect of parthenogenetic activation. *Exp. Cell Res.,* **32**: 168–170.

Burgos, M. H., 1955. The Feulgen reaction in mature unfertilized sea urchin eggs. *Exp. Cell Res.,* **9**: 360–363.

Carden, G. A., S. Rosenkranz and H. S. Rosenkranz, 1965. Deoxyribonucleic acids of sperm, eggs and somatic cells of the sea urchin *Arbacia punctulata. Nature,* **205**: 1338–1339.

Chamberlin, M., and P. Berg, 1962. Deoxyribonucleic acid-directed synthesis of ribonucleic acid by an enzyme from *Escherichia coli. Proc. Nat. Acad. Sci.,* **48**: 81–94.

Chandler, B., M. Hayashi, M. N. Hayashi and S. Spiegelman, 1964. Circularity of the replicating form of a single-stranded DNA virus. *Science,* **143**: 47–49.

Chèvremont, M., 1962. Localization and synthesis of deoxyribonucleic acids in the cytoplasm of somatic cells of vertebrates: the role of mitochondria. *Biochem. J.,* **85**: 25P–26P.

Corneo, G., C. Moore, D. R. Sanadi, L. I. Grossman and J. Marmur, 1966. Mitochondrial DNA in yeast and some mammalian species. *Science,* **151**: 687–689.

Dawid, I. B., 1965. Deoxyribonucleic acid in amphibian eggs. *J. Mol. Biol.,* **12**: 581–599.

Dawid, I. B., 1966. Evidence for the mitochondrial origin of frog egg cytoplasmic DNA. *Proc. Nat. Acad. Sci.,* **56**: 269–276.

Denny, P. C., and A. Tyler, 1964. Activation of protein biosynthesis in non-nucleate fragments of sea urchin eggs. *Biochem. Biophys. Res. Commun.,* **14**: 245–249.

Dische, von Z., 1930. Über einige neue charakteristische Farbreaktionen der Thymonukleinsäure und eine Mikromethode zur Bestimmung derselben in tierischen Organen mit Hilfe dieser Reaktionen. *Mikrochemie,* **2** (Neue Folge): 4–32.

Eberhard, A., and D. Mazia, 1965. Conditions for extraction of cytoplasmic DNA from embryos of a sea urchin. *Biochem. Biophys. Res. Commun.,* **21**: 460–465.

Elson, D., T. Gustafson and E. Chargaff, 1954. The nucleic acids of the sea urchin during embryonic development. *J. Biol. Chem.,* **209**: 285–294.

GIBOR, A., AND S. GRANICK, 1964. Plastids and mitochondria: Inheritable systems. *Science,* **145**: 890–897.

GIUDICE, G., 1960. Incorporation of labeled amino acids into the proteins of the mitochondria isolated from the unfertilized eggs and developmental stages of *Paracentrotus lividus. Exp. Cell Res.*, **21**: 222–225.

GIUDICE, G., AND A. MONROY, 1958. Incorporation of S^{35}-methionine in the proteins of the mitochondria of developing and parthenogenetically activated sea urchin eggs. *Acta Embryologiae et Morphologiae Experimentalis,* **2**: 58–65.

GRANT, P., 1965. Informational molecules and embryonic development. *In:* The Biochemistry of Animal Development. R. Weber, Editor. Academic Press, New York, pp. 488–593.

GROSS, P. R., AND G. H. COUSINEAU, 1963. Effects of actinomycin D on macromolecule synthesis and early development in sea urchin eggs. *Biochem. Biophys. Res. Commun.*, **4**: 321–326.

GROSS, P. R., AND G. H. COUSINEAU, 1964. Macromolecule synthesis and the influence of actinomycin on early development. *Exp. Cell Res.*, **33**: 368–395.

HAGGIS, A. J., 1964. Quantitative determination of deoxyribonucleic acid in embryos and unfertilized eggs of *Rana pipiens. Devel. Biol.*, **10**: 358–377.

HINEGARDNER, R. T., 1961. The DNA content of isolated sea urchin egg nuclei. *Exp. Cell Res.*, **25**: 341–347.

HOFF-JØRGENSEN, E., 1954. Deoxynucleic acid in some gametes and embryos. *In:* Recent Developments in Cell Physiology. J. A. Kitching, Editor. Colston Papers, **7**: 79–90.

HOTTA, Y., AND A. BASSEL, 1965. Molecular size and circularity of DNA in cells of mammals and higher plants. *Proc. Nat. Acad. Sci.,* **53**: 356–362.

HULTIN, T., 1961. Activation of ribosomes in sea urchin eggs in response to fertilization. *Exp. Cell Res.*, **25**: 405–417.

KALF, G. F., 1964. Deoxyribonucleic acid in mitochondria and its role in protein synthesis. *Biochemistry*, **3**: 1702–1706.

KAY, E. R. M., 1964. Direct isolation of deoxyribonucleic acid from cells. *Nature*, **202**: 390–391.

KLEINSCHMIDT, A. K., A. BURTON AND R. L. SINSHEIMER, 1963. Electron microscopy of the replicative form of the DNA of the bacteriophage ØX174. *Science*, **142**: 961.

KLEINSCHMIDT, A. K., S. J. KASS, R. C. WILLIAMS AND C. A. KNIGHT, 1965. Cyclic DNA of Shope papilloma virus. *J. Mol. Biol.*, **13**: 749–756.

KROON, A. M., 1963. Inhibitors of mitochondrial protein synthesis. *Biochim. Biophys. Acta*, **76**: 165–167.

LANZAVECCHIA, G., 1960. The yolk formation in the frog oocytes. Electron microscope studies. Symposium on the Germ Cells and Earliest Stages of Development, Pallanza. S. Ranzi, Editor. Inst. Internatl. d'Embryologie and Fondazione A. Baselli, Milan, pp. 61–74.

LANZAVECCHIA, G., 1965. Structure and demolition of yolk in *Rana esculenta* L. *J. Ultrastr. Res.*, **12**: 147–159.

LEIF, R. C., AND J. VINOGRAD, 1964. The distribution of buoyant density of human erythrocytes in bovine albumin solutions. *Proc. Nat. Acad. Sci.,* **51**: 520–528.

LUCK, D. J. L., AND E. REICH, 1964. DNA in mitochondria of *Neurospora crassa. Proc. Nat. Acad. Sci.,* **52**: 931–938.

MARMUR, J., AND P. DOTY, 1962. Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J. Mol. Biol.,* **5**: 109–118.

MARSHAK, A., AND C. MARSHAK, 1953. Desoxyribonucleic acid in *Arbacia* eggs. *Exp. Cell Res.*, **5**: 288–300.

MEZGER-FREED, L., 1963. Evidence for synthesis of deoxyribonucleic acid in enucleated *Rana pipiens* eggs. *J. Cell Biol.,* **18**: 471–475.

MONROY, A., 1965. Chemistry and Physiology of Fertilization. Holt, Rinehart and Winston, New York.

MOUNOLOU, J. C., H. JAKOB AND P. P. SLONIMSKI, 1966. Mitochondrial DNA from yeast "petite" mutants: Specific changes in buoyant density corresponding to different cytoplasmic mutations. *Biochem. Biophys. Res. Commun.*, **24**: 218–224.

NAKANO, E., AND A. MONROY, 1958. Incorporation of S^{35}-methionine in the cell fractions of sea urchin eggs and embryos. *Exp. Cell Res.,* **14**: 236–243.

NASS, M. M. K., 1966. The circularity of mitochondrial DNA. *Proc. Nat. Acad. Sci.,* **56**: 1215–1222.

NASS, M. M. K., S. NASS AND B. A. AFZELIUS, 1965. The general occurrence of mitochondrial DNA. *Exp. Cell Res.,* **37**: 516–539.

NASS, S., AND M. M. K. NASS, 1963. Intramitochondrial fibers with DNA characteristics. II. Enzymatic and other hydrolytic treatments. *J. Cell Biol.,* **19**: 613–629.

OGUR, E., AND G. ROSEN, 1950. The nucleic acids of plant tissues. I. The extraction and estimation of desoxypentose nucleic acid and pentose nucleic acid. *Arch. Biochem.,* **25**: 262–276.

PIKÓ, L., AND A. TYLER, 1965. Deoxyribonucleic acid content of unfertilized sea urchin eggs. *Amer. Zool.,* **5**: 636.

RABINOWITZ, M., J. SINCLAIR, L. DE SALLE, R. HASELKORN AND H. SWIFT, 1965. Isolation of deoxyribonucleic acid from mitochondria of chick embryo heart and liver. *Proc. Nat. Acad. Sci.,* **53**: 1126–1133.

RIS, H., 1962. Ultrastructure of certain self-dependent cytoplasmic organelles. Proceedings of the 5th International Congress of Electron Microscopy, Vol. 2. Academic Press, New York, pp. xx–l.

ROLLER, A., 1963. Étude de l'acide désoxyribonucléique des oeufs de Batraciens: teneur et localisation. *Arch. Intern. Physiol. Bioch.,* **71**: 139–140.

RUDZINSKA, M. M., P. A. D'ALESANDRO AND W. TRAGER, 1964. The fine structure of *Leishmania donovani* and the role of the kinetoplast in the Leishmania-leptomonad transformation. *J. Protozool.,* **11**: 166–191.

SCHATZ, G., E. HALSBRUNNER AND H. TUPPY, 1964a. Deoxyribonucleic acid associated with yeast mitochondria. *Biochem. Biophys. Res. Commun.,* **15**: 127–132.

SCHATZ, G., E. HALSBRUNNER AND H. TUPPY, 1964b. Intramitochondriale Deoxyribonukleinsäure in Säugetierzellen. *Monatshefte für Chemie,* **95**: 1135–1140.

SCHILDKRAUT, C. L., AND S. LIFSEN, 1965. Dependence of the melting temperature of DNA on salt concentration. *Biopolymers,* **3**: 195–208.

SCHILDKRAUT, C. L., J. MARMUR AND P. DOTY, 1962. Determination of base composition of deoxyribonucleic acid from its buoyant density in CsCl. *J. Mol. Biol.,* **4**: 430–443.

SCHNEIDER, W. C., AND E. L. KUFF, 1965. The isolation and some properties of rat liver mitochondrial deoxyribonucleic acid. *Proc. Nat. Acad. Sci.,* **54**: 1650–1658.

SEGOVIA, Z. M. M., F. SOKOL, I. L. GRAVES AND W. W. ACKERMANN, 1965. Some properties of nucleic acids extracted with phenol. *Biochim. Biophys. Acta,* **95**: 329–340.

SHAVER, J. R., 1956. Mitochondrial populations during development of the sea urchin. *Exp. Cell Res.,* **11**: 548–559.

SHAVER, J., 1957. Some observations on cytoplasmic particles in early echinoderm development. *In:* The Beginnings of Embryonic Development. A. Tyler, C. B. Metz and R. C. von Borstel, Editors. American Association for the Advancement of Science, Washington, D. C., pp. 263-290.

SHMERLING, ZH. G., 1965. Isolation and properties of DNA of sturgeon oocytes. *Biokhimiya,* **30**: 113–123 (in Russian); translation **30**: 96–104.

SINCLAIR, J. H., AND B. J. STEVENS, 1966. Circular DNA filaments from mouse mitochondria. *Proc. Nat. Acad. Sci.,* **56**: 508–514.

SRIVASTAVA, M. D. L., 1965. Cytoplasmic inclusions in oogenesis. *Internat. Rev. Cytol.,* **18**: 73–98.

STEINERT, G., H. FIRKET AND M. STEINERT, 1958. Synthèse d'acide désoxyribonucléique dans le corps parabasal de *Trypanosoma mega*. *Exp. Cell Res.,* **15**: 632–635.

SUGINO, Y., N. SUGINO, R. OKAZAKI AND T. OKAZAKI, 1960. Studies on deoxynucleoside compounds. I. A modified microbioassay method and its application to sea urchin eggs and several other materials. *Biochim. Biophys. Acta,* **40**: 417–424.

SUYAMA, Y., AND J. R. PREER, JR., 1965. Mitochondrial DNA from protozoa. *Genetics,* **52**: 1051–1058.

SWIFT, H., 1965. Nucleic acids of mitochondria and chloroplasts. *Amer. Naturalist,* **99**: 201–227.

SWIFT, H., N. KISLEV AND L. BOGORAD, 1964. Evidence for DNA and RNA in mitochondria and chloroplasts. *J. Cell Biol.,* **23**: 91A.

TYLER, A., 1963. The manipulations of macromolecular substances during fertilization and early development of animal eggs. *Amer. Zool.,* **3**: 109–126.

TYLER, A., 1965. The biology and chemistry of fertilization. *Amer. Naturalist,* **99**: 309–334.

TYLER, A., 1966. Incorporation of amino acids into protein by artificially activated non-nucleate fragments of sea urchin eggs. *Biol. Bull.,* **130**: 450–461.

TYLER, A., AND B. S. TYLER, 1966a. The gametes: Some procedures and properties. *In:* Physiology of Echinodermata, Ch. 27. R. A. Boolootian, Editor. John Wiley and Sons, Inc., New York, pp. 639–682.

TYLER, A., AND B. S. TYLER, 1966b. Physiology of fertilization and early development. *In:* Physiology of Echinodermata, Ch. 28. R. A. Boolootian, Editor. John Wiley and Sons, Inc., New York, pp. 683–741.

VAN BRUGGEN, E. F. J., P. BORST, G. J. C. M. RUTTENBERG, M. GRUBER AND A. M. KROON, 1966. Circular mitochondrial DNA. *Biochim. Biophys. Acta,* **119**: 437–439.

VINOGRAD, J., AND J. HEARST, 1962. Equilibrium sedimentation of macromolecules and viruses in a density gradient. *Fortschr. Chem. Org. Naturstoffe,* **22**: 372–422. Springer-Verlag, Vienna.

VINOGRAD, J., AND J. LEBOWITZ, 1966. Physical and topological properties of circular DNA. *J. Gen. Physiol.,* **49**: 103–125.

VINOGRAD, J., J. LEBOWITZ, R. RADLOFF, R. WATSON AND P. LAIPIS, 1965. The twisted circular form of polyoma viral DNA. *Proc. Nat. Acad. Sci.,* **53**: 1104–1111.

WARD, R. T., 1962. The origin of protein and fatty yolk in *Rana pipiens.* II. Electron microscopical and cytochemical observations of young and mature oocytes. *J. Cell Biol.,* **14**: 309–341.

WEIL, R., AND J. VINOGRAD, 1963. The cyclic helix and cyclic coil forms of polyoma viral DNA. *Proc. Nat. Acad. Sci.,* **50**: 730–738.

WEISS, S. B., AND L. GLADSTONE, 1959. A mammalian system for the incorporation of cytidine triphosphate into ribonucleic acid. *J. Amer. Chem. Soc.,* **81**: 4118–4119.

WHITELEY, A. H., AND F. BALTZER, 1958. Development, respiratory rate and content of desoxyribonucleic acid in the hybrid *Paracentrotus* ♀ × *Arbacia* ♂. *Pubbl. Staz. Zool. Napoli,* **30**: 402–457.

OBSERVATIONS ON THE GENITAL SEGMENTS OF SPIRORBIS (POLYCHAETA)[1]

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The serpulid genus *Spirorbis* has attracted the attention of numerous investigators for more than a century. Pagenstecher (1863) noted that *Sp. pagenstecheri* exhibited brood protection and was hermaphroditic. In subsequent accounts (Agassiz, 1866; Fewkes, 1885; Schively, 1897; Bush, 1904; Elsler, 1907; zur Loye, 1908; Borg, 1917; and others) Pagenstecher's observations have been confirmed for all species examined. To date, the most comprehensive study on the morphology and reproductive biology of *Spirorbis* is that of Bergan (1953a). Bergan's study, although contributing valuable information, leaves many questions unanswered and fails to give an adequate description of the genital segments. The observations presented here were made in conjunction with an embryological study of *Spirorbis* and serve to add new information concerning the morphology and nature of the genital segments in this complex and enigmatic genus.

Materials and Methods

Collection and maintenance of adult animals

Spirorbis (*Laeospira*) *mörchi* Levinsen, *Sp.* (*Paradexiospira*) *vitreus* Fabricius, and *Sp.* (*Dexiospira*) *spirillum* Linné were collected intertidally on San Juan Island, Washington, periodically throughout the year, from 1960 to 1963. *Spirorbis spirillum* was also frequently collected on hydroid colonies (*Abietinaria* sp.) dredged 10–23 fathoms off San Juan Island. *Spirorbis* (*Protolaeospira*) *ambilateralis* Pixell was collected by dredging off San Juan Island and was found most frequently on *Modiolus modiolus*, *Balanus nubilis*, and *Chlamys* sp., and often in association with *Sp. vitreus*. The system of classification followed is that first used by Caullery and Mesnil (1897) and later adopted by Fauvel (1927). The identities of the species reported on here were determined from descriptions given by Bush (1904), Pixell (1912), Fauvel (1927), Berkeley and Berkeley (1952), Bergan (1953b), and Pettibone (1954). In an examination of the Washington species, the procedure was first to prepare a species description, as complete as possible, before going to the literature to make comparisons. Of the several hundreds of specimens examined, *Sp. mörchi* and *Sp. ambilateralis* have always been found to be sinistral and *Sp. spirillum* dextral. On the west side of San Juan Island, Washington, however, there are extensive populations of dextral and sinistral *Sp. vitreus*. The sinistral form occurs together with the dextral form in about

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[1] Supported, in part, by predoctoral fellowship 1-F1-GM-20, 593-01 from U.S.P.H.S.

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equal numbers. They are exactly alike in all diagnostic characters except the direction of coil. Observations on isolated brooding adults indicate that this is a case of true genetic polymorphism (Potswald, 1965).

The animals were kept in the circulating sea water system at the Friday Harbor Laboratories, or were brought to the Seattle campus where they were kept in the Zoology Department's 10° C. cold room. In Seattle, no attempt was made to provide food; however, the animals remained in good condition for several months at a time, and brooding individuals were always available.

Adult animals were observed both within their tubes and removed from their tubes. In the latter case, animals were removed from their substratum by means of a razor blade and then removed from their tubes by chipping away the calcareous secretion with dissecting needles. This is a relatively easy task except for *Sp. vitreus,* which has an extremely hard tube.

Microtechnique

Adult worms removed from their tubes were fixed in a variety of fixatives including: Bouin's fluid, Helly's with and without post-chroming, buffered formalin, Flemming's with and without acetic acid, and Carnoy without chloroform. Fixed material was dehydrated through ethyl alcohol and tertiary butyl alcohol, and embedded in a mixture of 300 gm. of Fisher's Tissuemat (M.P. 60–62° C.) and 45 gm of dry piccolyte. This is the mixture recommended by Cloney (1961) but without beeswax. Blocks, chilled in ice-water, were sectioned 5–6 microns at room temperature.

A number of stains were used including Heidenhain's iron haematoxylin, Heidenhain's Azan, and Harris' or Ehrlich's haematoxylin with eosin counterstain. Feulgen and PAS methods were followed according to McManus and Mowry (1960).

In addition to routine paraffin technique, material was fixed in cold buffered osmium tetroxide (Bennett and Luft, 1959) and embedded in Epon 812 according to the method of Luft (1961). Thin sections, $\frac{1}{2}$–1 micron, were cut on a Porter-Blum ultra-microtome, using glass knives. After affixing to glass slides, the sections were stained with Richardson's stain (Richardson *et al.,* 1960) for study with the light microscope.

DESCRIPTION OF GENITAL SEGMENTS

Bergan (1953a), from his study of *Sp. borealis,* concluded that *Spirorbis* is a simultaneous hermaphrodite and not protandrous as suggested by Hempelmann (1931). Although providing histological evidence for his conclusion, Bergan fails to give an adequate description of the genital segments. Aside from Franzén's study (1956, 1958) on late stages of spermiogenesis and Dasgupta and Austin's (1960) examination of chromosome numbers in spermatocytes, little information concerning the gametes and their development in *Spirorbis* is available.

The observations presented here are based, for the most part, on a study of *Sp. mörchi.* Only where differences in detail have been found, will the other three species be mentioned.

Female Segments

Normal arrangement and anomalies

Germ cell proliferation is restricted to the abdominal or secondary segments; the thoracic or primary segments never serve in this capacity. In a mature adult every abdominal segment contains germ cells in various stages of formation. Each genital segment contains a large coelomic space and is separated from its neighbors by complete septa. In *Sp. mörchi* the first two abdominal segments are female and the remaining abdominal segments male (Fig. 2). *Sp. ambilateralis* presents the same arrangement, but the left halves of segments one and two are generally larger than the right halves of the same segments. Of the dextral species, *Sp. vitreus* is like *Sp. mörchi*, while *Sp. spirillum* presents quite a different picture. In local populations of *Sp. spirillum*, either the first three segments or first 3½ segments are female; the left half of the fourth segment in the latter condition is male. The anomalous condition is the most prevalent. According to Bergan (1953a), specimens of *Sp. spirillum* collected in Oslofjord exhibit lateral asymmetry in sex differentiation similar to that described above; therefore, it would seem that such anomalous sex differentiation is common in *Sp. spirillum*.

Although one might expect to find anomalies in a simultaneous hermaphrodite such as *Spirorbis*, sex differentiation within the genus, with the notable exception of *Sp. spirillum*, seems to be under rigid control and a specific arrangement of female and male segments prevails for a given species. After examination of several hundred specimens of adult *Sp. mörchi*, only a few anomalies have been observed. Lateral asymmetry was found in one animal in which the left half of the second segment was male and the right half female. One case was observed in which the first three abdominal segments were female instead of the first two as is the normal condition. Finally, in a few instances, individuals were found to have sperm and oocytes developing together in the same segment. The latter condition generally occurs in the posterior male segments where the infrequent oocytes have never been observed to enter into vitellogenesis. Only in two individuals have oocytes and sperm been observed to develop together in the second abdominal segment, between a purely female and a male segment (Fig. 3). Oocytes developing in the posterior part of the achaetous zone, as described by Bergan (1953a) for *Sp. borealis*, have not been found.

Development of the primary oocyte

The gonad is a discrete and constant organ composed of clumps of cells arranged in two retroperitoneal rows, mesial to the ventral nerve cords, and running the length of the abdominal segments (Figs. 4, 5, 6, 7). In sexually mature adults, the gonad is always larger in the female segments than in the male segments where it is greatly compressed against the ventral ectoderm.

An examination of the gonad in the female segments reveals the presence of a number of primordial germ cells in interphase. While in interphase, these are the most distinctive cells in the adult body. The cells have almost spherical nuclei 5 to 6 microns in diameter, and the cytoplasm is reduced to a mere envelope. Scattered around the periphery of the nucleus, in a predictable pattern, are spherical clumps of Feulgen-positive chromatin; the center of the nucleus resists staining and appears transparent (Fig. 1a). Counts, made from thick sections, indicate that there are ten chromatin clumps per nucleus, this number apparently being constant. A nucleolus is absent. The cytoplasm is so reduced that little detail can be resolved in it with light microscopy.

Since the gonad is the site of constant proliferation, the primordial germ cells are generally observed in various stages of mitosis. As nuclei enter prophase, the thread-like projections appear to radiate out from the individual chromatin spheres and the "unraveling" continues until the nucleus is completely occupied by typical prophase chromosomes (Fig. 1a). Metaphase, anaphase, and telophase follow in an orderly fashion. The telophase nuclei of the resulting daughter cells are small and vesicular, with the diameter of the nuclei measuring about 2.5 microns and that of the entire cell about 3.2 microns (Fig. 1b). It is assumed that the mitotic divisions give rise either to more primordial germ cells which resume the characteristic interphase condition or to a morphologically different cell type, the early primary oocyte. The term "primordial germ cell" has been used for the proliferating cell since a distinct gonial stage has not been recognized.

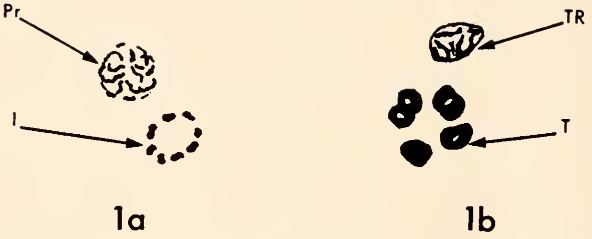


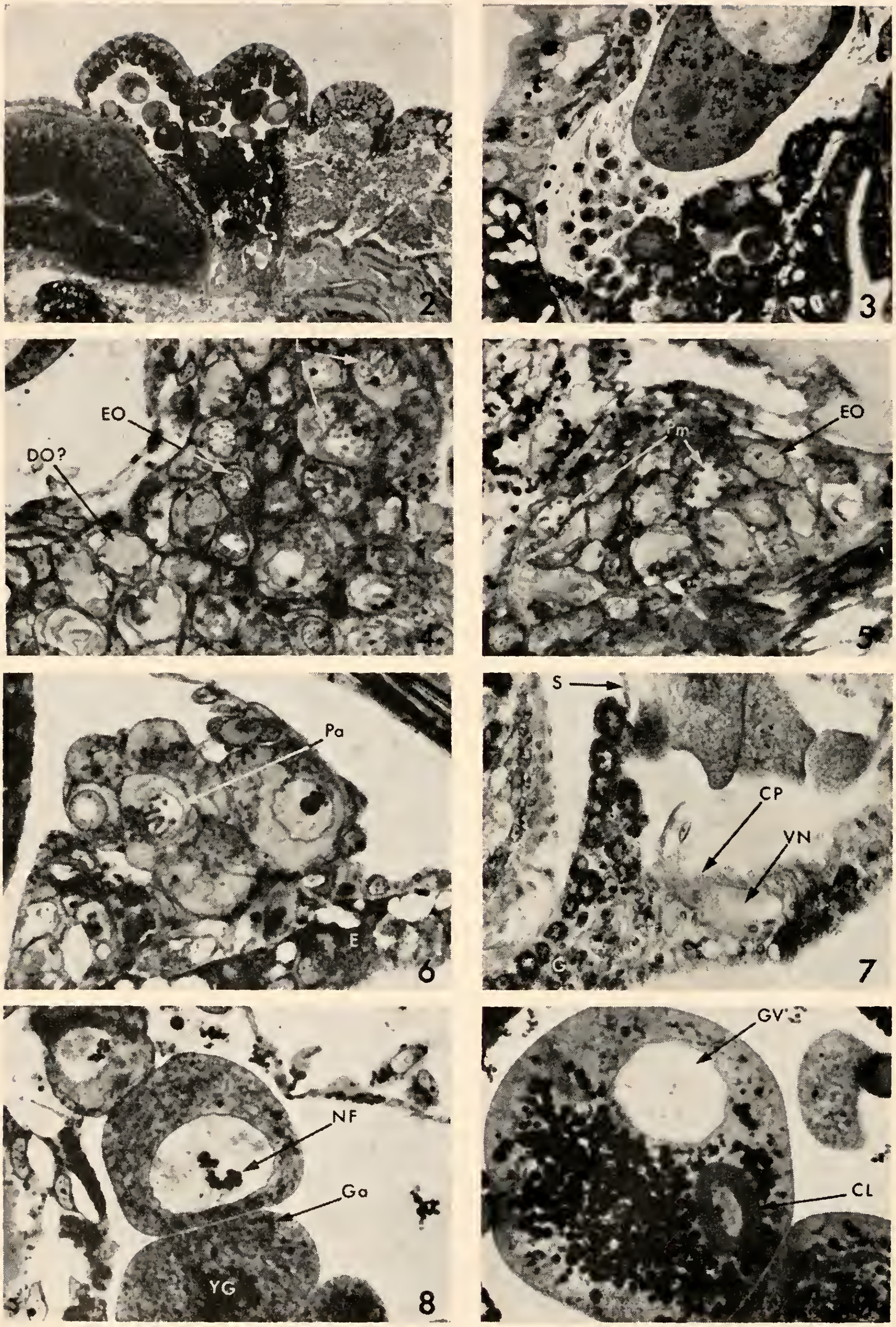
FIGURE 1. *Sp. mörchi:* (1a) section through primordial germ cells in interphase (I), and prophase (Pr); (1b) section through four primordial germ cells having just completed telophase (T), and one primordial germ cell undergoing telophase reconstruction (TR). Drawing made from a photomicrograph. Paraffin; Feulgen. (1880 ×)

Early primary oocytes have a nuclear diameter of about 6 microns and a cytoplasmic component somewhat larger than that found in the primordial germ cell. The nucleus contains a single nucleolus and a diffuse chromatin network (Figs. 4, 5). Premeiotic stages of prophase are passed within the gonad proper. The delicate chromatin net of the interphase nucleus becomes coarser and more irregular and, with further condensation, chromatin clumps appear not unlike those found in the primordial germ cell. The nucleus has now become elliptical in shape and measures about 7.5 microns in length (Fig. 5). Following condensation, the chromatin bodies become frayed and give rise to delicate chromosomal threads. As concerns succeeding stages of premeiotic prophase, only leptotene and early pachytene, still in "bouquet" arrangement, have been observed (Figs. 4, 6). Scattered throughout the gonad are a number of cells about the same size as oocytes in meiotic prophase; however, unlike the normal oocytes, these cells have structureless nuclei often irregular in outline (Fig. 4). The role of the latter cells in normal development is not clear, but it is possible that they are degenerating germ cells.

With the conclusion of the events of premeiotic prophase, the nucleus again enters the diffuse or "confused" state and regains its spherical shape. A distinct nucleolus reappears along with several irregularly shaped pieces of chromatin; the basophilic cytoplasmic portion has increased and the diameter of the cell is now about 11 microns. Oocytes of the latter size-class are found beneath the bulging peritoneal covering of the gonad and at the base of the septa. As the oocytes increase in size, they invade the septum and a progressive series can be found extending dorsally from the gonad. The oocytes are located between the two thin epithelial layers making up the septum (Fig. 7). While within the septum, the cytoplasm of the oocytes is strongly basophilic, and little or no yolk deposition occurs. In nuclei attaining a diameter of 13 microns, the single nucleolus starts to fragment into a number of small Feulgen-negative bodies. Cells having this nuclear diameter are about 22 microns in diameter and are starting to erupt into the coelom. Their cytoplasm is filled with coarse membrane, a few scattered proteid yolk granules, and numerous, small, spherical mitochondria (Fig. 8). In thin sections of osmium-fixed material, small rod-like bodies about one micron long, staining intensely with Richardson's stain, are found in the cytoplasm around the nucleus. The bodies are often crescent-shaped and may represent Golgi material (Fig. 8).

The bulk of vitellogenesis takes place in oocytes which have erupted through the septum, and are floating free within the spacious coelom. There are no nurse cells, as such, nor follicle cells associated with the oocytes. As cytoplasmic mass increases, nuclei, which also steadily increase in size, become more eccentric in position. In cells measuring 39 × 33 microns and having a nuclear diameter of about 22 microns, yolk granules begin to arise in a rather localized area within the cytoplasm. At the start of vitellogenesis, and even at its close, yolk granules give only a slight PAS reaction which is not qualitatively affected by incubation in diastase. Associated with the yolk granules are several lamellar stacks of membrane. The membrane stacks are very sensitive to the fixative employed and have been observed only in material fixed in either buffered osmium or Helly's fluid followed by post-chroming. As oocytes approach a diameter of about 50 microns, the lamellar stacks become concentric, thereby enclosing an internum. Clusters of mitochondria are associated with the concentric bodies, and both proteid and lipid yolk granules occur within the internum (Fig. 9). The description given here corresponds in many respects to those given by several authors for structures termed "yolk nuclei," which have been observed in various groups of animals (see Raven, 1961, for survey). As vitellogenesis proceeds and the cytoplasm becomes filled with yolk granules, the concentric structures disappear; their fate has not been followed. There is little visible differentiation of the cortex below the PAS-positive vitelline membrane, cortical granules apparently being absent.

Maximum growth has been attained, and the primary oocyte is ready to be spawned, when it measures about 165 microns × 132 microns. From the start of vitellogenesis, yolk granules have increased in diameter from a fraction of a micron, in the case of proteid yolk, to about 8 microns. The germinal vesicle has become very wrinkled in appearance and has an average measurement of 26 × 13 microns. Nucleolar fragments, so abundant during vitellogenesis, have become reduced both in number and in size. The germinal vesicle apparently does not break down until after spawning has occurred. Animals ready to spawn contain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CL

FIGURES 2–9.

two size-classes of oocytes in their coeloms: full grown primary oocytes and early primary oocytes still within or attached to the septa. Animals have been observed to spawn within 12 hours after releasing larvae, and since it takes about 30 days from fertilization to larval release at 12° C., it is assumed that vitellogenesis occupies about the same period of time.

From the present study, it is difficult to come to any definite conclusions as to the origin of yolk. Nucleolar fragmentation, concentric lamellae, and possible Golgi material have been mentioned, and it is conceivable that they all participate in yolk formation. At the onset of vitellogenesis, the peritoneal cells lining the coelomic cavity start to accumulate large droplets, both lipoidal and proteid in nature, within their cytoplasm. The droplets accumulate to such a degree as to cause the ordinarily flattened peritoneal cells to bulge into the coelomic cavity (Fig. 10). Apical portions of cells actually bud off and become free in the coelom. Towards the end of vitellogenesis, the peritoneal cells again become flattened and relatively devoid of inclusions. There is no evidence that the oocytes are phagocytic; however, correlation between the onset and decline of the storage phenomenon in peritoneal cells with that of vitellogenesis would seem to suggest that transfer of material in some form takes place. Conceivably, such transfer could be in the form of high molecular weight compounds. Finally, it should be mentioned that it is not uncommon for one or two oocytes per segment to disintegrate midway through vitellogenesis. It is not known what role, if any, this phenomenon plays during normal development of the primary oocyte.

The above outline of events leading to the development of the primary oocyte in *Sp. mörchi* also holds true, in the main, for the other three species studied. In *Sp. vitreus,* the behavior of the nucleolus is quite different from that in *Sp. mörchi.*

FIGURE 2. *Sp. mörchi:* parasagittal section through the abdomen of a typical adult, showing that the first two abdominal segments are female and the remaining abdominal segments are male. Epon; Richardson's stain. (110 ×)

FIGURE 3. *Sp. mörchi:* parasagittal section through the second abdominal segment of an adult, showing the simultaneous development of oocytes and spermatids within the same segment. Epon; Richardson's stain. (700 ×)

FIGURE 4. *Sp. mörchi:* frontal section through the gonad in a female segment showing presumably degenerating oocytes (DO?), early oocytes (EO), and leptotene (L). Epon; Richardson's stain. (800 ×)

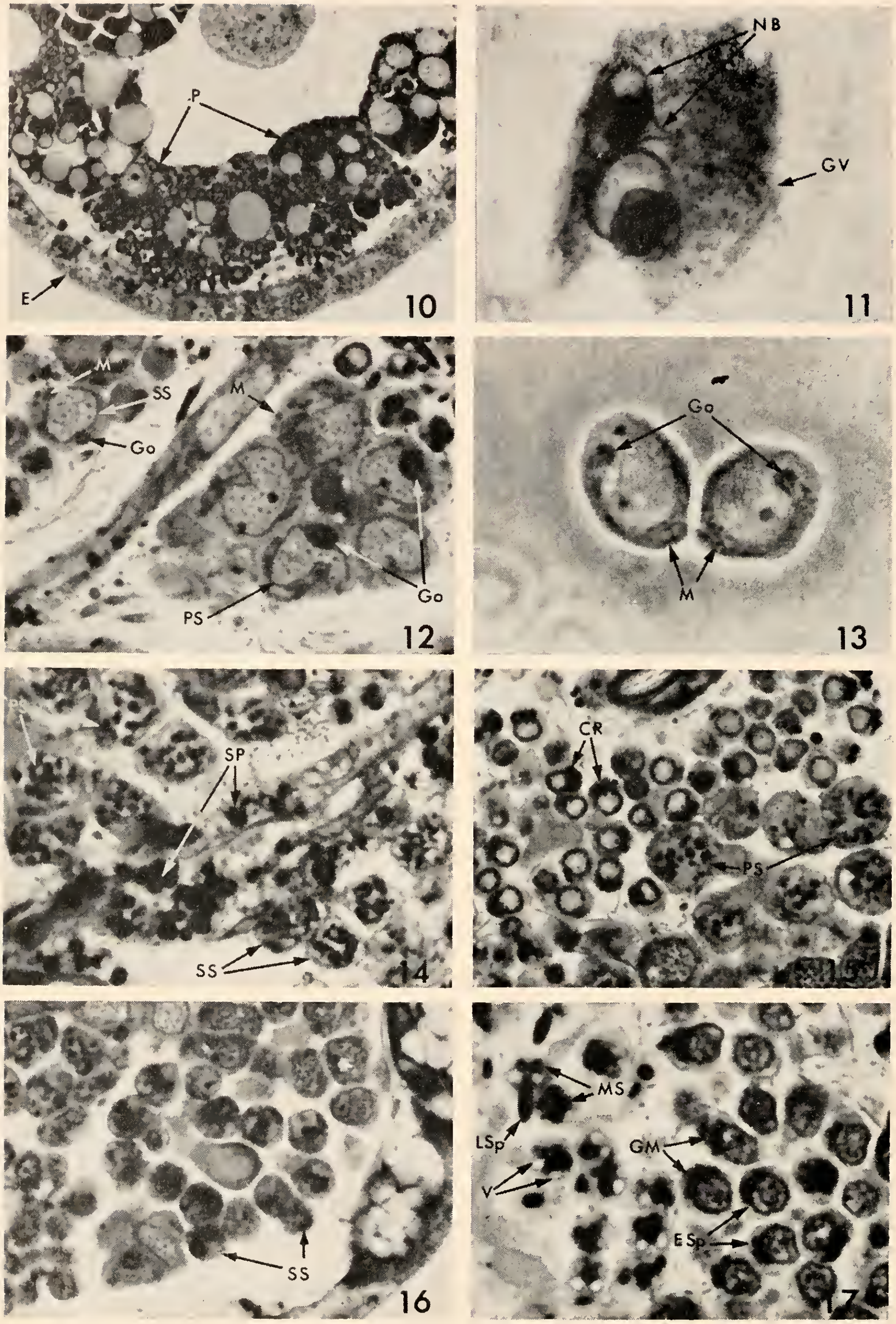
FIGURE 5. *Sp. mörchi:* frontal section through the gonad of a female segment showing early oocytes (EO) and a premeiotic oocyte (Pm) containing condensed, peripheral chromatin. Epon; Richardson's stain. (800 ×)

FIGURE 6. *Sp. mörchi:* cross-section through the gonad of a female segment showing an oocyte in pachytene (Pa). Note the discrete nature of the gonad and the thin ventral epidermis (E). Epon; Richardson's stain. (800 ×)

FIGURE 7. *Sp. mörchi:* oblique cross-section through the achaetous region and first abdominal segment showing a progressive series of oocytes extending dorsally from the gonad (G) within the septum (S). Also illustrated are the ventral nerve cord (VN) and ventral ciliated peritoneum (CP). Paraffin; haematoxylin-eosin. (320 ×)

FIGURE 8. *Sp. mörchi:* oblique cross-section through two adjacent female segments showing a series of oocytes which have erupted through the septum into the coelom. Note the large amount of coarse membrane present in the cytoplasm, the nucleolar fragments (NF), and possible Golgi material (Go). Only a few proteid yolk granules (YG) are present. Epon; Richardson's stain. (800 ×)

FIGURE 9. *Sp. mörchi:* cross-section through an oocyte showing the eccentric position of the germinal vesicle (GV) and concentric lamellae (CL) of membrane. Note absence of follicle cells. Epon; Richardson's stain. (800 ×)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Figures 10–17.

The nucleolus in early oocytes is homogeneous, but just prior to vitellogenesis it takes on the staining characteristics of an amphinucleolus. The cortex is acidophilic and the medulla basophilic. As yolk granules begin to appear, the cortex separates into two spherical bodies which retain their identity throughout most of the vitellogenetic period. The medulla also divides and starts to vacuolate and extrude nucleolar material into the nucleoplasm (Fig. 11). Nucleolar vacuolation also occurs in *Sp. spirillum*. The nucleolar products in both species can be observed in germinal vesicles about to be spawned. At the level of light microscopy, there is no evidence for nucleolar extrusion into the cytoplasm.

Bergan (1953a), although he does not describe them, claims to have found abdominal nephridia in the female segments of the four species he examined. He is of the opinion that the dimensions of the nephridia are such that they could serve as genital ducts. It has not been possible to confirm Bergan's observations on local species used in the present study. In *Sp. mörchi,* for example, the ventral peritoneum of the female segments is strongly ciliated but there is no duct arrangement (Fig. 7). This ciliated patch of peritoneum probably represents a remnant of the coelomostome, the habit of shedding oocytes through a coelomoduct having been abandoned. Such remnants as a ciliated flap or patch on the peritoneum are common in polychaetes which release gametes by rupture of the body wall or gut (Dales, 1963). In *Sp. mörchi*, gravid female segments become greatly distended, the ventral body wall measuring about 8 microns in thickness.

Male Segments

Spermatocyte development

The mitotic events associated with primordial germ cell proliferation in the male gonad are the same as in the female gonad. As in the female gonad, pre-

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FIGURE 10. *Sp. mörchi:* parasagittal section through a female segment showing the large accumulation of lipid droplets within the peritoneal cells (P) and the thin epidermis (E) of the body wall. Epon; Richardson's stain. (630 ×)

FIGURE 11. *Sp. vitreus:* section through a primary oocyte showing the germinal vesicle (GV) and nucleolar fragments undergoing nucleolar vacuolation (NB). Paraffin; haematoxylin-eosin. (1600 ×)

FIGURE 12. *Sp. mörchi:* sagittal section through two adjacent male segments showing a cluster of primary spermatocytes (PS) and a secondary spermatocyte (SS). Note the spherical Golgi material (Go) and mitochondria (M). Epon; Richardson's stain. (1600 ×)

FIGURE 13. *Sp. vitreus:* primary spermatocytes showing spherical Golgi material (Go) and cluster of mitochondria (M) at the opposite pole. Living material; phase-contrast. (1600 ×)

FIGURE 14. *Sp. mörchi:* sagittal section through two adjacent male segments showing primary spermatocytes (PS) just prior to first meiotic metaphase and secondary spermatocytes (SS) just prior to second meiotic metaphase. Note inclusions in septal peritoneum (SP). Epon; Richardson's stain. (1600 ×)

FIGURE 15. *Sp. mörchi:* section through a male segment showing primary spermatocytes (PS) in first meiotic metaphase and spermatids in the "complete ring stage" (CR). Epon; Richardson's stain. (1260 ×)

FIGURE 16. *Sp. mörchi:* section through a male segment showing secondary spermatocytes (SS) in second meiotic metaphase. Epon; Richardson's stain. (1260 ×)

FIGURE 17. *Sp. mörchi:* section through a male segment showing early spermatids (ESp) and late spermatids (LSp). Note the granular mitochondria (GM) concentrated at one pole in the early spermatids and fusion of mitochondria to form four mitochondrial spheres (MS) in the late spermatids. The four vacuoles (V) found in the sloughing cytoplasm of the late spermatids alternate with the four mitochondrial spheres. Epon; Richardson's stain. (1925 ×)

meiotic stages of prophase are difficult to find, only leptotene having been observed on the male side. Also, as in the female gonad, there are a number of cells which have the appearance of cells undergoing degeneration; however, as in the case of the female side, there is no clear evidence that these cells actually degenerate. The primary spermatocyte erupts through the peritoneum of the septum and enters the coelom with the nucleus in the "diffuse state." The fact that growth of the primary oocyte is initiated in the gonad probably accounts for the disparity in size of gonad between the two sexes.

In the coelom, the primary spermatocyte reaches a diameter of about 8 microns. The nucleus contains a fine chromatin network and a single nucleolus. Thread-like mitochondria are scattered throughout the cytoplasm but are especially concentrated at one pole of the cell. At the opposite pole there is a spherical structure which, in osmium-fixed material, is partially or completely surrounded by a sheath of dark-staining material (Fig. 12). The same structure has also been observed in living primary spermatocytes by means of phase-contrast (Fig. 13). It is assumed that the spherical body is made up of Golgi material. Just prior to metaphase I, the chromosomes appear as distinct bivalents and the spherical body breaks up (Fig. 14). The diplotene chromosomes contract greatly as they enter diakinesis but unequivocal chiasma formations have not been observed. With the conclusion of diakinesis, the chromosomes become arranged in metaphase (Fig. 15) and the first meiotic division proceeds. Counts made from sectioned material and aceto-orcein squashes indicate the haploid number to be about 10. Dasgupta and Austin (1960) in five species of *Spirorbis* (*Sp. borealis, Sp. corallinae, Sp. pagenstecheri, Sp. spirillum,* and *Sp. tridentatus*) have found a uniform count of $2n = 20$.

Secondary spermatocytes, resulting from the first meiotic division, enter into an interphase condition. The single nucleolus and delicate chromatin network reappear in the nucleus and the entire diameter of the cell averages 5.7 microns. Thread-like mitochondria remain more or less concentrated at one pole while at the opposite pole dense-staining bodies reaggregate to form the spherical body which is assumed to be of Golgi material (Fig. 12). With the onset of prophase, the nucleolus disappears and prophase chromosomes appear (Fig. 14). After contracting to some extent, the chromosomes become arranged along the metaphase plane and the second meiotic division occurs (Fig. 16).

Spermiogenesis

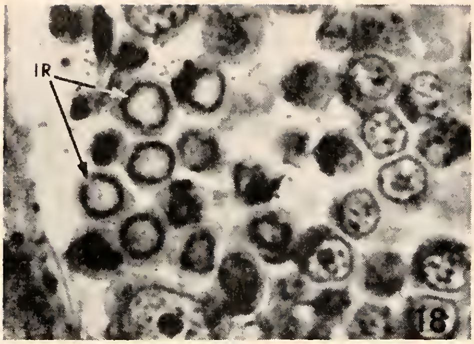
The early spermatid resulting from telophase II has a total diameter of 3.3 microns. A delicate chromatin net fills the nucleus and granular mitochondria are concentrated at one pole within the scanty cytoplasm (Fig. 17). With the initiation of spermiogenesis, the chromatin goes through a series of complex changes. At the beginning of the cycle, the chromatin pulls away from the center of the nucleus and starts to condense around the periphery just under the nuclear membrane. Further peripheral condensation gives rise to what may be referred to as the "interrupted ring stage." The mitochondria in the latter stage are still granular in appearance and concentrated at one pole (Fig. 18). The next stage in the developmental sequence is characterized by completion of the chromatin ring and is referred to here as the "complete ring stage." At this stage, the mitochondria have fused into four spheres of uniform size which surround the point from which the tail filament emerges (Fig. 15).

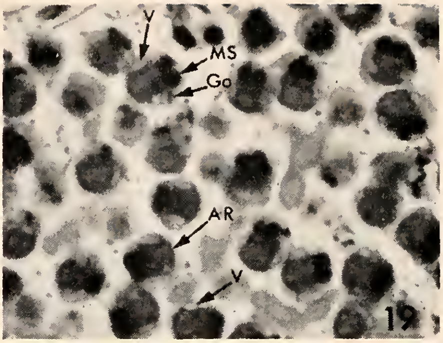
In material fixed in buffered osmium and stained with Richardson's stain, the chromatin ring stains very intensely while the center of the nucleus stains hardly at all. With further development, the chromatin ring disappears except for a dark-staining apical rim, and the nucleus becomes homogeneous. It has not been possible to resolve the events involved in acrosome formation; however, in the last mentioned stage, a dark-staining body is found in the cytoplasm at one side of the developing sperm head and may represent Golgi material (Fig. 19). The dark-staining apical rim might be interpreted to be the deposited acrosome. After becoming homogeneous, the nucleus takes on a conical shape and starts to elongate (Fig. 20). As the head continues to elongate, the cytoplasm moves backward so as to encompass more and more of the tail filament. Four clear vacuoles appear in the apical portion of the sloughing cytoplasm just above the mitochondrial spheres. A cross-section taken through this region of the developing sperm reveals that the vacuoles alternate with the four mitochondrial spheres (Fig. 17). The vacuoles occur in all four species examined and are undoubtedly the "neutral red vacuoles" described by Franzén (1956). The significance of the vacuoles is not known.

Late spermatids in various stages of cytoplasm elimination form large plates when artificially released into the sea water. This seems to be due to a mutual stickiness and not to the presence of a cytophore or nurse cell arrangement; at least if such a system is present, it is not resolved at the light level. In the last phase of cytoplasm elimination, the four mitochondrial spheres move posteriorly along the tail filament to form the middle piece. In mature sperm, the middle piece becomes homogeneous. The vacuoles are lost with the residual cytoplasm.

The morphologically mature sperm of *Sp. mörchi* has a cylindrical head capped with a distinctly pointed acrosome and has a total length of about 45 microns. Sharply delimited from the head is the middle piece which is about three times the length of the head. The tail filament is of the ordinary type and is just a little more than twice the length of the middle piece (Fig. 21). In *Sp. ambilateralis* the head of the sperm is also cylindrical but the acrosome is neither as distinct nor as pointed as in *Sp. mörchi.* The middle piece is 1½ times the length of the head and the tail filament has a length equal to the total length of a *Sp. mörchi* sperm (Fig. 22). The average total length of a *Sp. ambilateralis* sperm is 57 microns.

In the two dextral species, *Sp. vitreus* and *Sp. spirillum,* the sperm are morphologically quite different from those of the two sinistral species just described. In both the dextral form and polymorphic sinistral form of *Sp. vitreus,* the sperm head has the shape of a long, slightly bent, pointed cone, a middle piece slightly less in length but of about the same thickness as the head, and a tail filament with a length a little more than twice the combined length of head and middle piece (Fig. 23). The sperm head of *Sp. spirillum* is longer, more sharply bent, and more pointed than that of *Sp. vitreus.* The middle piece is similar but the tail filament is considerably longer, having a length equal to that of an entire *Sp. vitreus* sperm (Fig. 24). In neither species is the acrosome clearly delimited from the head. The average total length of a *Sp. vitreus* sperm is 42.5 microns, whereas

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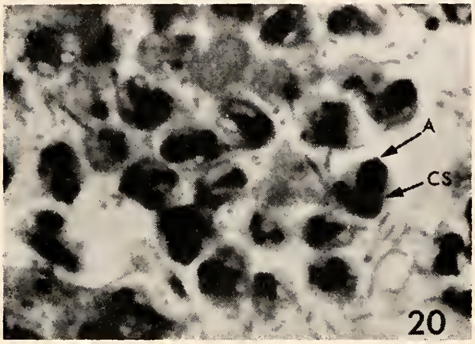

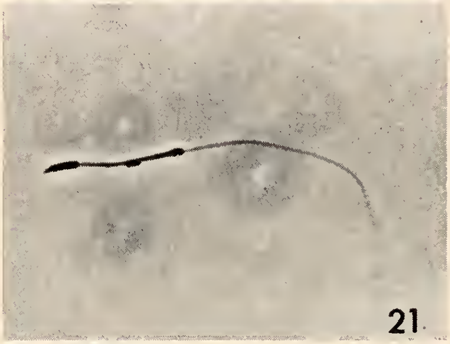

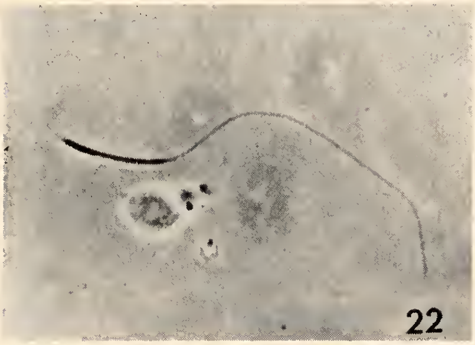

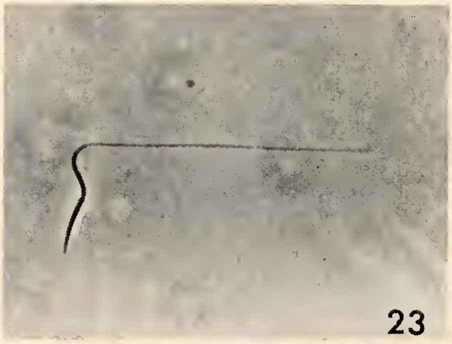

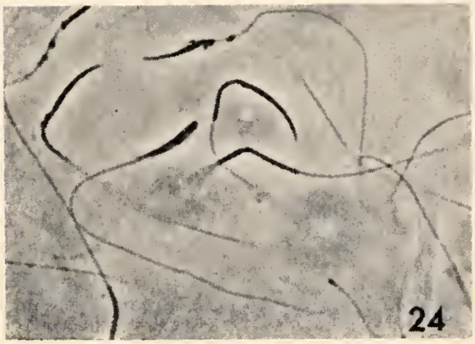

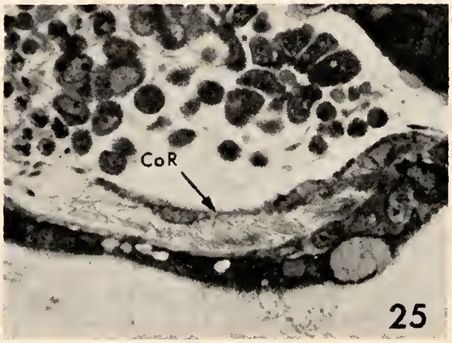

FIGURES 18–25.

the average length of a *Sp. spirillum* sperm is 59 microns. The descriptions for the dextral species are essentially in agreement with those given by Franzén (1956).

Arrangement of stages within the male segments

Each male segment in a sexually mature animal contains stages of spermiogenesis. Synchronous stages are found in clusters within the coelom, but there is no predictable arrangement and each segment is autonomous. Generally, a single segment contains clusters of primary spermatocytes, secondary spermatocytes, and spermatids; however, a single segment may or may not also contain meiotic figures and mature sperm. As one would expect, the synchronous clusters of primary spermatocytes are smallest in size, the cluster size reaching a maximum with the spermatids and sperm. To account for this arrangement, there must be a simultaneous proliferation of primary spermatocytes occurring periodically in each male segment throughout the sexual period.

There is no clear evidence, at the level of light microscopy, for the existence of nurse cells in male segments. It is interesting to note, however, that the peritoneal cells lining the coelomic cavity accumulate inclusions not unlike those found in the peritoneal cells of the female segments (Fig. 14). This accumulation of inclusion bodies never reaches the degree observed in female segments.

The septa separating the male segments are, like those of the female segments, made up of two thin epithelial layers of peritoneal origin. In each half of the male segments, the peritoneal layer of the posterior septum is folded in upon itself in such a manner as to form a short duct near the ventral floor of the coelom. Each duct, the ventral portion of which is ciliated, ends blindly at the same level and just lateral to the ventral nerve cords (Fig. 25). Mature spermatozoa are often found in the ducts, but stages of spermatogenesis from primary spermatocytes to late spermatids may also be found in the ducts. Bergan (1953a) refers to these as abdominal nephridia but this can hardly be correct since their peritoneal origin is obvious. It seems most likely that the ciliated ducts represent remnant coelomostomes.

DISCUSSION

The most striking feature concerning the secondary segments of *Spirorbis* is the fact that each functions as a genital segment and contains a well defined and

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FIGURE 18. *Sp. mörchi:* section through a male segment showing spermatids in the "incomplete ring stage" (IR). Epon; Richardson's stain. (1925 ×)

FIGURE 19. *Sp. mörchi:* section through a male segment showing spermatids in the "homogeneous stage." Note the apical rim (AR), mitochondrial spheres (MS), forming vacuoles (V), and dark-staining body which may be Golgi rest (Go). Epon; Richardson's stain. (1925 ×)

FIGURE 20. *Sp. mörchi:* section through a male segment showing spermatids in the "conical stage" (CS). Note that the acrosome (A) is now visible. Epon; Richardson's stain. (1925 ×)

FIGURE 21. *Sp. mörchi:* nearly mature sperm. Middle piece still somewhat irregular in appearance. Living material; phase-contrast. (1140 ×)

FIGURE 22. *Sp. ambilateralis:* mature sperm. Living material; phase-contrast. (1140 ×)

FIGURE 23. *Sp. vitreus:* mature sperm. Living material; phase-contrast. (1140 ×)

FIGURE 24. *Sp. spirillum:* mature sperm. Living material; phase-contrast. (1140 ×)

FIGURE 25. *Sp. mörchi:* cross-section through a male segment showing a coelomostome rudiment (CoR). Epon; Richardson's stain. (700 ×)

persistent gonad. Distinct gonads have been described in *Salmacina dysteri* (Malaquin, 1925), *Filograna implexa* (Faulkner, 1930), *Pomatoceros triqueter* (Thomas, 1940; Jyssum, 1957), and in the Arenicolidae (Fauvel, 1959; Matthews, 1962). With the exception of the forms just mentioned, a discrete gonad is not characteristic of polychaetes. In a majority of polychaetes the germ cells arise from rather indefinite patches of peritoneum (Parker and Haswell, 1957; Fauvel, 1959; Dales, 1963).

Cells having features similar to those of the primordial germ cells in *Spirorbis* have been reported in the three serpulid species mentioned above (Malaquin, 1925; Faulkner, 1930; Jyssum, 1957). Jyssum (1957) in a study on oogenesis in *Pomatoceros triqueter* refers to these cells as neoblasts and describes them as giving rise to the female gamete. The interphase nuclei of the neoblasts contain peripheral chromatin clumps; however, they are not as regular in size and form as are those of the primordial germ cells in *Spirorbis*. Another difference between the two is that the neoblasts contain one or two nucleoli. Jyssum describes the neoblasts as dividing and giving rise to gonia which, in turn, divide to give rise to primary oocytes. The distinction between the two divisions is apparently based on the thickness of the chromosomes at metaphase, the chromosomes of the neoblasts being thicker and more lumpy than the chromosomes in gonial metaphase. Such a gonial stage, between primordial germ cell and primary cyte, has not been observed in *Spirorbis*. A distinct gonial stage in *Spirorbis* is quite probable, but, as yet, has not been identified. It will be shown, in a subsequent report, that the primordial germ cells arise relatively early in the development of *Spirorbis* and can be followed to the sites of gonad formation in the metamorphosed pre-adult.

Many of the problems associated with oogenesis have already been discussed. The fact that most of the growth and maturation of the oocyte occur freely within the coelom without the aid of attached follicle or nurse cells offers a number of possibilities for experimental investigation of this type of oocyte development. For example, it may be possible to culture oocytes *in vitro* and study the effects of environmental conditions on growth and vitellogenesis.

There are a number of problems associated with spermiogenesis in *Spirorbis* which are beyond the resolution afforded by the light microscope. It would be of interest, for example, to study acrosome formation. Only a few electron microscope studies of acrosome formation in invertebrates have been attempted (see Cameron and Fogal, 1963) and some of the homologies between various types of acrosomes are at a start of being elucidated. Another problem which would be of interest is the origin and possible significance of the "neutral red vacuoles" which are so apparent during spermiogenesis.

The mature sperm of *Spirorbis* is not of the simple or primitive type but is modified. Two morphological types have been described here. Franzén (1956) recognizes three morphological types in the genus: *Sp. spirillum* and *Sp. vitreus* share one type; *Sp. borealis* and *Sp. granulatus* share another type quite different from the first; and a third type, even more highly modified than the other two, is found in *Sp. pagenstecheri*. The descriptions given in the present study for *Sp. spirillum* and *Sp. vitreus* agree in the main with those given for the same species by Franzén. Sperm morphology of *Sp. mörchi* and *Sp. ambilateralis* is not consistent with any of the three types recognized by Franzén. There can be no doubt that the sperm morphology in the genus *Spirorbis* has some usefulness as an auxiliary systematic character. In this connection it may again be pointed out that the sperm morphology of the sinistral form of *Sp. vitreus* is identical with that of the dextral form.

Franzén's major thesis is that there is a definite relation between the morphology of the sperm and the biology of fertilization. According to this thesis, invertebrates which discharge their gametes freely into the water retain a primitive type of sperm which is characterized as consisting of a short rounded to conical head, a small middle piece containing four to five mitochondrial spheres, and a tail formed by a long flagellum. Invertebrates which have an altered biology of propagation exhibit a modified sperm morphology. If the end product of spermatogenesis is a modified sperm, primitive characters are retained during spermiogenesis; the four mitochondrial spheres which appear in the spermatid of *Spirorbis* would be such a character. In a discussion of the family Serpulidae, Franzén points out the hermaphroditic and brooding nature of *Spirorbis* but admits that literature on the reproductive biology of the genus is extremely incomplete. Speculating on the mode of fertilization, he is of the opinion that the most natural way for it to occur would be that sperm from a nearby animal are sucked into the tube and there fertilize the eggs. If this were the case, sperm would not have to swim great distances in order to reach the eggs. There is also evidence that at least certain species of *Spirorbis* are capable of self-fertilization (Potswald, 1964; Gee and Williams, 1965).

The writer wishes to express his gratitude to Dr. Robert L. Fernald, Director of the Friday Harbor Laboratories, for his valuable advice and assistance. Drs. W. Siang Hsu and Paul L. Illg are thanked for their helpful suggestions.

SUMMARY

1. In all species of *Spirorbis* examined, the first two or three abdominal segments of mature adults are female and the remaining abdominal segments are male. Both female and male gametes differentiate simultaneously in the same individual and arise from a discrete and persistent gonad composed of primordial germ cells arranged in two retroperitoneal rows, mesial to the ventral nerve cords, and running the length of the abdominal segments.

2. Cytological events associated with the development of female and male gametes are described. Differentiation of oocytes and spermatocytes occurs freely within the coelomic cavity without the aid of attached nurse cells.

3. Although coelomostome rudiments are present in both female and male segments, functional genital ducts do not develop. Spawning is assumed to take place by rupture of the body wall.

LITERATURE CITED

AGASSIZ, A., 1866. On the young stages of a few annelids. *Ann. Lyceum Nat. Hist. N. Y.*, 7: 303–343.

BENNETT, H. S., AND J. LUFT, 1959. S-collidine as a basis for buffering fixatives. *J. Biophys. Biochem. Cytol.*, 6: 113–117.

BERGAN, P., 1953a. On the anatomy and reproductive biology of *Spirorbis* Daudin. *Nytt Magasin Zool. Oslo*, **1**: 1-26.

BERGAN, P., 1953b. The Norwegian species of *Spirorbis* Daudin. *Nytt Magasin Zool. Oslo*, **1**: 27–48.

BERKELEY, E., AND C. BERKELEY, 1952. Polychaeta sedentaria. *Canad. Pacific Fauna*, **9b**: 132–138.

BORG, F., 1917. Über die Spirorbisarten Schwedens. *Zool. Bidrag Uppsala*, **5**: 15–38.

BUSH, K. J., 1904. Tubiculous annelids of the tribes Sabellides and Serpulides from the Pacific Ocean. *Harriman Alaska Exped.*, **12**: 169–298.

CAMERON, M. L., AND W. FOGAL, 1963. The development and structure of the acrosome in the sperm of *Lumbricus terrestris* L. *Canad. J. Zool.*, **41**: 753–761.

CAULLERY, M., AND F. MESNIL, 1897. Études sur la morphologie comparée et la phylogenie des espèces chez les *Spirorbis*. *Bull. Sci. France Belgique*, **30**: 185–233.

CLONEY, R. A., 1961. Observations on the mechanism of tail resorption in ascidians. *Amer. Zool.*, **1**: 67–87.

DALES, R. P., 1963. Annelids. Hutchinson and Co., London.

DASGUPTA, S., AND A. P. AUSTIN, 1960. Chromosome numbers in serpulids. *Quart. J. Micr. Sci.*, **101**: 395–399.

ELSLER, E., 1907. Deckel und Brutpflege bei *Spirorbis*. *Zeitschr. wiss. Zool.*, **87**: 603–642.

FAULKNER, G. H., 1930. On the anatomy and histology of bud formation in *Filograna implexa*. *J. Linn. Soc. London (Zool.)*, **37**: 109–190.

FAUVEL, P., 1927. Polychètes sedentaires. *Faune de France, Paris*, **16**: 388–405.

FAUVEL, P., 1959. Classes des annélides polychètes. *Traité de Zoologie*, **5**: 132.

FEWKES, J. W., 1885. On the larval forms of *Spirorbis borealis*. *Amer. Naturalist*, **19**: 247–257.

FRANZÉN, Å., 1956. On spermiogenesis, morphology of the spermatozoon, and biology of fertilization among invertebrates. *Zool. Bidrag Uppsala*, **31**: 355–482.

FRANZÉN, Å., 1958. On sperm morphology and acrosome filament formation in some Annelida, Echiuroidea, and Tunicata. *Zool. Bidrag Uppsala*, **33**: 1–28.

GEE, J. M., AND G. B. WILLIAMS, 1965. Self and cross-fertilization in *Spirorbis borealis* and *S. pagenstecheri*. *J. Mar. Biol. Assoc.*, **45**: 275–285.

HEMPELMANN, F., 1931. Archiannelida + Polychaeta. *Handbuch der Zoologie*, **2**: 90.

JYSSUM, S., 1957. Investigations of the neoblasts and oogenesis in the serpulid *Pomatoceros triqueter* L. *Nytt Magasin Zool. Oslo*, **5**: 5–10.

LOYE, J. F. ZUR, 1908. Die Anatomie von *Spirorbis borealis* mit besonderer Berücksichtigung der Unregalmässigkeiten des Körperbau und deren Ursachen. *Zool. Jahrb. Abt. Anat. Ontog. Tiere*, **26**: 305–354.

LUFT, J. H., 1961. Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.*, **9**: 409–414.

MALAQUIN, A., 1925. Les cellules germinales (gonocytes) sont, au cours de la reproduction asexuée de *Salmacina dysteri* Huxley, la source de la proliferation blastogénique. *C. R. Acad. Sci. Paris*, **180**: 873–875.

MATTHEWS, L. H., 1962. The structure of the ovary. A. Invertebrates. *In:* volume I, The Ovary, edited by Sir Solly Zuckerman. Academic Press, New York.

McMANUS, J. F. A., AND R. W. MOWRY, 1960. Staining Methods Histologic and Histochemical. Paul B. Hoeber, Inc., New York.

PAGENSTECHER, H. A., 1863. Untersuchungen über niedere Seetiere aus Cett. VII. Entwickelungsgeschichte und Brutpflege von *Spirorbis spirillum*. *Zeitschr. wiss. Zool.*, **12**: 486–496.

PARKER, T. J., AND W. A. HASWELL, 1957. A Text-book of Zoology. I. 6th ed. Macmillan Co., London.

PETTIBONE, M. H., 1954. Marine polychaete worms from Point Barrow, Alaska, with additional records from the North Atlantic and North Pacific. *Proc. U. S. Nat. Mus.*, **103**: 341–345.

PIXELL, H., 1912. Polychaeta from the Pacific Coast of North America. Part I. Serpulidae with a revised table of classification of the genus *Spirorbis*. *Proc. Zool. Soc. London*, **2**: 784–805.

POTSWALD, H. E., 1964. The nature of the primordial germ cells and evidence for self-fertilization in *Spirorbis* (Polychaeta, Serpulidae). *Amer. Zool.*, **4**: Abstract 93.

POTSWALD, H. E., 1965. Reproductive biology and development of *Spirorbis* (Polychaeta, Serpulidae). Doctoral Thesis, University of Washington, Dissertation Abstracts No. 65-8528.

RAVEN, CHR. P., 1961. Oogenesis: The Storage of Developmental Information. Pergamon Press, New York.

RICHARDSON, K. C., L. JARETT AND E. H. FINKE, 1960. Embedding in epoxy resins for ultrathin sectioning in electron microscopy. *Stain Technol.*, **35**: 313–323.

SCHIVELY, M. A., 1897. The anatomy and development of *Spirorbis borealis*. *Proc. Acad. Nat. Sci. Philadelphia*, **49**: 153–160.

THOMAS, J. G., 1940. *Pomatoceros, Sabella* and *Amphitrite*. Liverpool Mar. Biol. Committee, Memoir XXXIII.

CHOLINESTERASE IN THE BRAIN OF THE CECROPIA SILKMOTH DURING METAMORPHOSIS AND PUPAL DIAPAUSE[1]

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In giant silkmoths, pupal diapause results from the failure of neurosecretory elements in the brain and corpora cardiaca to provide the hormonal stimulus required for initiation of further development; the latter resumes, months later, when the neurosecretory system recovers its function after exposure of the pupa to appropriate temperatures and photoperiod (Williams, 1946, 1952, 1956; Williams and Adkisson, 1964). For further resolution of the control of diapause and development, a key problem is to define the physiological processes, within the brain, that establish and later efface its endocrine impotence.

As one aspect of this problem, we have examined the behavior of cholinesterase (ChE) in the brain of the Cecropia silkmoth. Our inquiry is based on the findings of Van der Kloot (1955), who reported a disappearance of ChE and spontaneous electrical activity in the brain at the time of pupation, and their subsequent reappearance together with the brain's recovery of endocrine activity. The changes were found to be limited to the brain, since the thoracic and abdominal ganglia retained normal levels of enzymatic and electrical activity throughout diapause. On the basis of the close temporal correlations observed, and the possible functional roles of ChE, Van der Kloot recognized that its behavior could account for the neuroendocrine inactivation and reactivation of the brain. As is apparent from current reviews on insect endocrinology and development (*e.g.,* Wigglesworth, 1964; Gilbert, 1964), these observations have remained the most promising leads to date on the control of neurosecretion and diapause in lepidopterous insects.

However, the aforementioned conclusions have recently been questioned as a result of two brief reports (Schoonhoven, 1963; Tyshtchenko and Mandelstam, 1965) containing electrophysiological observations supplemented by certain limited enzymatic data. The observations were made on diapausing pupae belonging to several families of Lepidoptera, and including a number of Cecropia. Since the electrophysiological findings described in these two papers conflict with one another, as well as with the observations of Van der Kloot, the net result has been to create a rather uncertain picture of the extent of neural activity during diapause. From the biochemical standpoint the picture is somewhat clearer but still equivocal. Through the use of a manometric technique, Schoonhoven detected hydrolytic activity in brains of diapausing pupae of a geometrid moth, *Bupalus piniarius* L.; Tyshtchenko and Mandelstam, by histochemical methods, detected hydrolytic activity in brains of diapausing pupae of the silkmoth *Antheraea pernyi* Guer. These activities were ascribed to ChE. Though rather suggestive of ChE, both reports omit mention of the biochemical details and controls required to confirm that the activity was in fact enzymatic, and that it was due to the action of ChE rather than that of other esterases known to occur in insect brain.

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[1] This study was supported, in part, by Public Health Service Research Grant GM-06101.

[2] Messrs. Eichenbaum and Locke held National Science Foundation Undergraduate Research Participation Awards during this investigation.

In the present study, we demonstrate by histochemical and quantitative methods that substantial ChE activity persists throughout diapause in brains of Cecropia and other silkmoths. Our findings provide comparative biochemical information on the properties and specificity of ChE in silkmoth brain, and show that diapause cannot be attributed to generalized absence of ChE, as seemed possible heretofore. But, for reasons to be discussed subsequently, the present findings do not preclude a regulatory role for one or more forms of esterase in the control of neurosecretion and diapause. These findings have been announced previously in abstract (Shappirio, Eichenbaum and Locke, 1965).

Materials and Methods

1. *Experimental animals*

Brains from the following species of silkmoths were used: *Hyalophora cecropia* (L.), *Samia cynthia* (Dru.), and *Antheraea polyphemus* (Cram.). For convenience, these will be identified henceforth as Cecropia, Cynthia, and Polyphemus, respectively. Most of the Cecropia were reared by us under nylon nets on wild cherry trees. Other Cecropia, as well as all the Cynthia and Polyphemus, were purchased from dealers in the northeastern and midwestern United States. The insects were managed as described previously (Williams, 1946; Shappirio and Williams, 1957). In addition to larvae and prepupae, we utilized: (a) "unchilled pupae" kept at 25° C., in which diapause was found to persist for more than five months after pupation; (b) "chilled pupae" stored at 6° C. to favor the prompt return of endocrine activity, followed by the resumption of development, upon their return to 25° C.; and (c) "developing adults" derived from previously chilled pupae allowed to terminate diapause at 25° C. Developing adults were staged by use of the morphological and physiological criteria summarized by Schneiderman and Williams (1954). In additional experiments, adult moths were used four days after their emergence at 25° C.

2. *Initial preparation of tissue*

For histochemical and quantitative studies, brains were excised from insects anesthetized in carbon dioxide (Williams, 1946) and then briefly rinsed in Ringer (Ephrussi and Beadle, 1936). In the case of larvae and prepupae, as well as diapausing pupae, the optic nerves were severed just distal to the melanin granules lying at their base; other nerves were severed as close as possible to the surface of the brain. In experiments on developing adults and adults, the optic lobes and tracts were included with the brain itself, but antennal and other nerves were transected at the surface of the brain and thus excluded from analysis. Also excluded was the subesophageal ganglion, by means of transverse cuts midway across both roots connecting this ganglion to the brain. Further details on tissue preparation are provided below.

3. *Histochemistry*

The size and fragile texture of silkmoth brains posed problems in tissue preparation which were sufficiently overcome only after extensive trials with various techniques. The methods giving most reliable results are described here. Brains were in certain cases prefixed in 10% formalin (pH 7.0) for 1–2 hours at 2° C., and then rinsed in Ringer for an equal time. Alternatively, postfixation was employed as a variant in technique and to control for possible formalin-induced fixation artifact; postfixation was accomplished by exposure of cryostat sections, mounted and sectioned as described below, to acetone at 2° C. for one hour before staining.

To provide a suitable matrix for sectioning, unfixed or fixed brains were embedded in small pieces of fresh mouse liver (approximately 5-mm. cubes) and immediately frozen in isopentane cooled to a viscous state by liquid nitrogen. The resulting frozen block was then sectioned in a Universal cryostat (−15° C.) at thicknesses of 8–10 μ. When possible, serial sections were placed in sequence on several microscope slides.

For detection of ChE activity in cryostat sections, we employed Gomori's (1952) modification of the Koelle (1951) method. In this procedure, acetylthiocholine (AThCh), a thioester analogous to acetylcholine (ACh), serves as substrate. Hydrolysis of the thioester yields thiocholine, which is precipitated at sites of reaction in the form of copper thiocholine sulfate; the latter is then converted to copper sulfide for easier visualization of reaction sites within the section. In our studies, an incubation time of 55–75 minutes at 20° C. proved optimal. After staining and conversion to copper sulfide, sections were lightly counterstained with aqueous Ehrlich hematoxylin, and mounted in glycerin jelly.

In histochemical studies involving inhibitors, cryostat sections were incubated for 30 minutes in Ringer containing the desired inhibitor concentration, before exposure to reaction medium which also contained inhibitor at this concentration. Control sections, serial when possible, were incubated and stained in parallel but without inhibitor.

4. *Quantitative enzymatic methods*

To obtain more detailed information on the properties of ChE in Cecropia brain, and to survey its behavior during the life history, we exploited the sensitive spectrophotometric method introduced by Ellman (Ellman *et al.*, 1961). This procedure also uses AThCh as substrate. Thiocholine generated by hydrolysis reacts with 5,5′-dithio-*bis*-2-nitrobenzoate (DTNB), incorporated in the reaction medium, to yield a bright yellow color attributable to the thionitrobenzoate anion. The reaction was followed at 412 mμ by means of a Beckman Model DU spectrophotometer. Rapid assay of ChE activity in individual brains was possible with this method.

In most experiments, each freshly excised brain was homogenized in a micro-size tissue grinder kept at 0° C., to yield 0.5 ml. of homogenate in 100 m*M* potassium phosphate, pH 8.0. This volume was adequate for duplicate or triplicate assays. In routine measurements of ChE activity in brains from animals at successive stages in the life history, we used a reaction volume of 1.02 ml. at 25° C., containing the following reagents at the final concentrations shown: 0.75 m*M* AThCh; 1.0 m*M* DTNB; 100 m*M* potassium phosphate, pH 8.0; and homogenate.

For other types of experiments, designed to examine the effects of pH, substrate concentration, and other factors, this protocol was modified as appropriate for the individual experiments noted under Results.

In experiments using inhibitors, the reaction was initially followed for 5 minutes in the absence of inhibitors, after which inhibitor was added to yield the desired final concentration. When the reaction had stabilized, which occurred within 5 minutes, the rate was recorded for a further 5 minutes. In all experiments, correction was made for changes in absorption due to endogenous thiols, which were very slight. Correction was also routinely made for changes in absorption due to non-enzymatic hydrolysis of substrate, which was 10% or less of the total reaction rate under routine conditions of assay described in the preceding paragraph. Suitable experiments established that the reaction rate, thus corrected, was proportional to the enzyme concentrations used, was linear during the period of assay, and was limited by the hydrolytic step of the reaction rather than by the steps involved in color development. Since pH 8.0 lies close to the limit of buffering action of the $HPO_4^{=}/H_2PO_4^{-}$ system, we also verified that this pH was maintained within 0.05 unit in the course of reactions at the most rapid rates encountered in this study.

5. *Chemicals*

AThCh and its homologues, propionylthiocholine and butyrylthiocholine (PrThCh and BuThCh, respectively), were purchased as the iodides from Sigma Chemical Co., St. Louis, Missouri. DTNB, eserine sulfate, and *tris*-(hydroxymethyl)-aminomethane (Tris buffer) were also Sigma products. The two Burroughs Wellcome anticholinesterase compounds, 62C47 and 284C51j, were generously furnished by Burroughs Wellcome, Inc., Tuckahoe, N. Y. These code names denote, respectively, 1,5-*bis*-(4-trimethylammoniumphenyl)-pentane-3-one diiodide, and 1,5-*bis*-(4-allyldimethylammoniumphenyl)-pentane-3-one dibromide. *Iso*-OMPA (tetraisopropyl pyrophosphortetramide) was obtained from Koch-Light Labs., Colnbrook, Bucks., England. Other chemicals were of analytical reagent grade.

RESULTS

1. *Histochemical observations*

Many general microanatomical features of the pupal brain in Cecropia are typical of the arthropod brains and central ganglia described in the treatise of Bullock and Horridge (1965). The pupal brain is bilobate in structure, with most cell bodies being located peripheral to large regions of neuropile. The latter consists of fine nerve fibers and cytoplasmic processes of glial cells. It constitutes the principal region of synaptic contact, and occupies substantial regions of the central, lateral, and ventral portions of each lobe, as well as the interior part of the tissue connecting the lobes.

When formalin-prefixed or acetone-postfixed cryostat sections were treated as described under Methods, an intense deposit of histochemical reaction product was observed in neuropile, as illustrated in Figure 1. Except for differences in fragmentation of tissue, which was greater after formalin treatment, prefixation and postfixation yielded an identical histochemical pattern. No reaction product was observed when AThCh was omitted from the reaction medium. Similar findings were made in each of 15 unchilled and chilled Cecropia pupae which were judged to be diapausing in terms of physiological and morphological criteria (Schneiderman and Williams, 1954).

With our material, the histochemical method did not afford sufficient resolution to permit us to ascertain the localization of ChE more intimately in the neuronal or glial elements of neuropile. However, in favorable preparations, reaction product was visualized in bands which apparently correspond to major prevailing directions of fiber tracts.

Initial insight into the specificity of the histochemical reaction in the neuropile was gained with two agents, eserine sulfate and *iso*-OMPA; the former is a general inhibitor of ChE's and the latter is relatively selective for mammalian butyrylcholinesterase (BuChE) when applied at appropriate concentrations. In brains of unchilled and chilled Cecropia pupae, the neuropile-associated esterase was found to be abolished by eserine sulfate at 10^{-5} M and to be unaffected by *iso*-OMPA at 10^{-4} M.

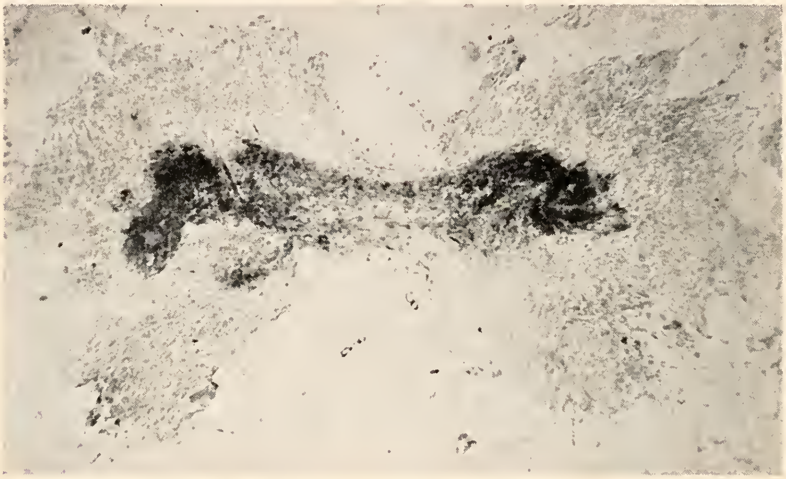

FIGURE 1. Section of brain from diapausing pupa, stained for ChE. The photograph depicts an 8 μ cryostat section, postfixed in acetone, which was stained histochemically using AThCh as substrate and counterstained with Ehrlich hematoxylin. The darkly stained region contains histochemical reaction product (CuS) and shows the localization of ChE activity (× 72).

Our observations on brains of post-diapausing Cecropia are limited in number, but permit several conclusions. When pupae chilled for 16 weeks were placed at 25° C., a period of 7–10 days elapsed before the first externally visible sign of adult development, namely, retraction of the leg epithelium from its overlying cuticle. This signals the second day of adult development (Schneiderman and Williams, 1954). In the present study, the histochemical pattern was found to remain identical with that encountered in brains of diapausing pupae, when observations were made during the first four or five days after transfer of the chilled pupae to 25° C. Subsequently, an increased area of deposition of reaction product was observed, accompanied by a slight though seemingly significant increased intensity of staining in neuropile. At the outset of adult development, esterase activity was no longer confined to neuropile, but had spread laterally and ventrally to encompass regions of neuronal or glial cell bodies. The esterase reaction in post-diapausing insects was inhibited fully by eserine sulfate at 10^{-5} *M*.

2. *Properties of ChE in pupal brain as revealed by quantitative methods*

In order to provide a meaningful basis for evaluating the behavior of ChE in relation to the onset and termination of pupal diapause, and to afford comparative biochemical insight, experiments were carried out to define optimal conditions of assay for ChE and to determine the specificity of the enzyme or enzymes detected.

a. *Effect of pH on ChE activity*

A series of measurements was performed on the same homogenate of brains pooled from unchilled diapausing pupae of Cecropia, using 100 m*M* phosphate or Tris–HCl buffers in the range pH 6.5 to 9.0. Although the activity with Tris was invariably lower than with phosphate (see below), in both cases enzyme activity increased from pH 6.5 to 8.0 but showed little increase in the range 8.0 to 9.0. For spectrophotometric assays, pH 8.0 was selected since this afforded a maximal rate with relatively low non-enzymatic hydrolysis of AThCh. The rate of the latter reaction increases markedly with further increase in pH, and at pH 9.0 exceeds the enzyme-catalyzed rate when the latter is kept within a range appropriate for meaningful assay. In experiments involving different pH's, suitable controls confirmed that the absorptivity of the thionitrobenzoate anion was essentially constant between pH 6.5 and 9.0.

b. *Effect of composition and concentration of certain buffers*

At pH 8.0, the reaction rate was found to be essentially similar when assays on the same homogenates were carried out in the presence of 100 m*M* phosphate or 70 m*M* bicarbonate (the latter charged at 25° C. with 5% carbon dioxide in oxygen). Only one-quarter of this rate was observed with 100 m*M* Tris at the same pH. The lower rate with Tris appears to be an inhibition rather than a failure of activation, since combination of phosphate and Tris did not elevate the rate above that observed with Tris alone. At 10 m*M*, the rate with phosphate was nearly 50% lower than at 100 m*M*. In view of these findings, a concentration of 100 m*M* was used in routine assays.

c. *Relationship of ChE activity to substrate concentration*

To the best of our knowledge, this important relationship has not been considered in previous studies on ChE in silkmoth brains. Figure 2 illustrates the striking dependence of ChE activity upon the concentration of the substrate, AThCh. A final substrate concentration of 0.75 m*M* was found to yield the most rapid reaction rate (Fig. 2). Above this concentration, one can readily observe that activity decreases progressively, yielding a graph of a shape typical for acetylcholinesterases (AChE's) in vertebrate and invertebrate preparations (Augustinsson, 1949, 1963).

In order to permit comparison between data obtained under conditions found optimal for assay of ChE in the present study, and the results of Van der Kloot's (1955) investigation, the substrate-activity curve for pupal brain ChE was also determined under our "routine" assay conditions, but supplemented by sodium chloride to yield a final concentration of 0.5 *M*. The latter had been employed in Van der Kloot's experiments owing to the findings of Chadwick *et al.* (1953) showing that it yielded optimal ChE activity in homogenates of brains from flies. Our experiments with Cecropia showed that the substrate-activity curves with and without extra salt possess similar shapes. However, the addition of salt alters

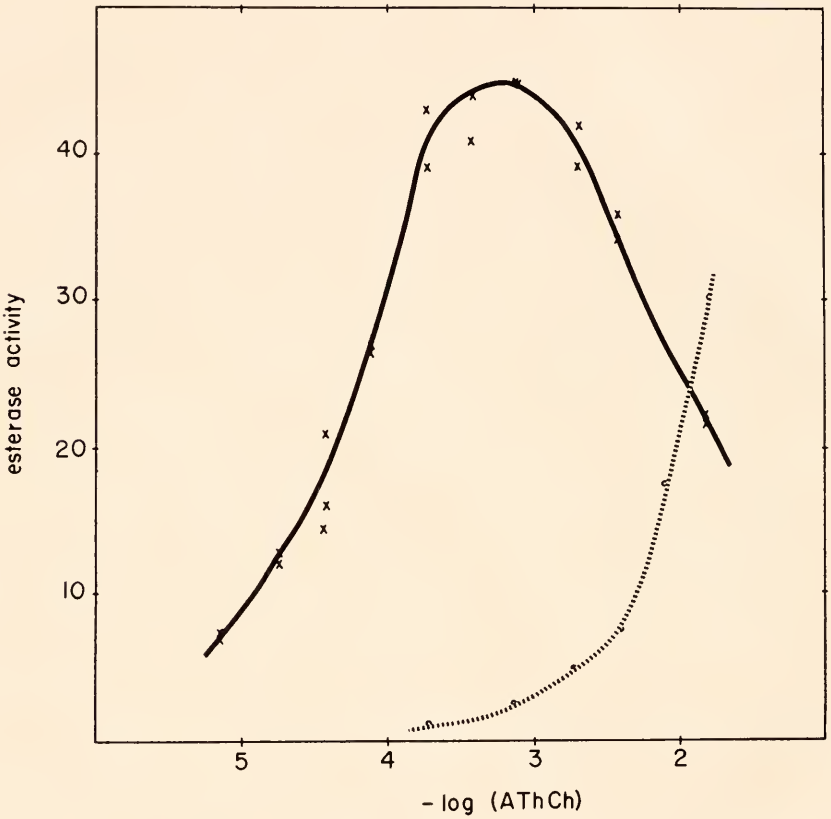

FIGURE 2. ChE activity as a function of AThCh concentration. The solid line describes enzymatic activity, which was corrected for spontaneous hydrolysis of AThCh at substrate concentrations above 10^{-4} *M*. The broken line describes the rate of spontaneous hydrolysis of AThCh. To provide sufficient material for the experiment illustrated, brains of six unchilled Cecropia pupae were pooled before homogenization. Enzyme activity is shown in relative units: one unit corresponds to a change in absorbance of 0.001 per minute.

both the optimal substrate concentration and the reaction rate at any given substrate concentration. In the presence of added salt, the substrate optimum was slightly higher, and when comparisons were made at the substrate optima with and without salt, that in salt was 22% lower.

d. *Effects of selective esterase inhibitors*

Information on the specificity of the ChE reaction in pupal brains was gained with a variety of agents known to be selective on the basis of studies with vertebrate and invertebrate preparations. The histochemical observations, described earlier, were confirmed by the finding that eserine sulfate at 10^{-5} *M* completely abolished enzymatic activity. The Burroughs Wellcome compounds, 62C47 and 284C51j, also inhibited the reaction fully when tested at final concentrations of 10^{-6} *M*. In contrast, *iso*-OMPA at 10^{-4} *M* failed to inhibit ChE in homogenates of brain from unchilled and chilled pupae.

Additional studies with inhibitors were carried out on brains from larvae, prepupae, developing adults, and adult moths. In all cases, eserine at 10^{-5} *M* was fully inhibitory. The agents 62C47 and 284C51j also fully inhibited ChE in larval brains and in brains from animals at the outset of adult development, when tested at 10^{-6} *M*. *Iso*-OMPA at 10^{-4} *M* was non-inhibitory in larvae, prepupae, and developing adults at early stages; but the brains of adult moths were slightly affected, a concentration of 10^{-4} *M* inhibiting their ChE by 8–10%.

e. *Reactivity of brain ChE with homologues of AThCh*

Figure 3 illustrates the relative reaction rates obtained at a graded series of concentrations of PrThCh and BuThCh. The homogenate used for the experiment illustrated, which gave typical results, was derived from the pooled brains of unchilled pupae. The same homogenate exhibited a rate of 24 (relative units as in Fig. 3) when examined with AThCh at 0.75 m*M*. Thus, the reaction rate decreases in the order: AThCh > PrThCh > BuThCh. Although only a limited range of concentrations was studied with the substrate homologues, it is clear that the substrate-activity curve for PrThCh resembles that for AThCh in showing a marked optimum substrate concentration, above which the reaction rate progressively decreases. Moreover, the position of the substrate optimum for PrThCh is rather close to that described (Fig. 2) for AThCh. In the range studied, reaction rate increased slightly with increase in BuThCh concentration, but showed no clear optimum. It was not possible to carry the studies with BuThCh to higher concentrations than are shown in Figure 3, inasmuch as the non-enzymatic breakdown of BuThCh became excessive when its concentration was increased further. The reactions with PrThCh and BuThCh were found to be fully sensitive to eserine at 10^{-5} M.

3. *ChE activity in Cecropia brain at successive stages in metamorphosis and during diapause*

In order to permit observations on as homogeneous a sample of insects as possible, ChE activity was determined in individuals of the same batch of Michigan-reared Cecropia. Our 1964 crop, used for this purpose, was especially suitable since the proportion of animals emerging precociously from pupal diapause was unusually low; only two individuals out of more than 800 initiated development and emerged as moths during the first 6 months after pupation, without prior chilling at 6° C.

Table I summarizes measurements of ChE activity in 46 individual brains from animals at successive stages in the life history, ranging from late in larval life through and beyond pupal diapause to the initial phase of adult development. All measurements were made under the routine conditions of assay described under Methods. As shown in the Table, ChE was readily detectable at all stages examined. Of special interest is the finding that ChE activity undergoes no decline

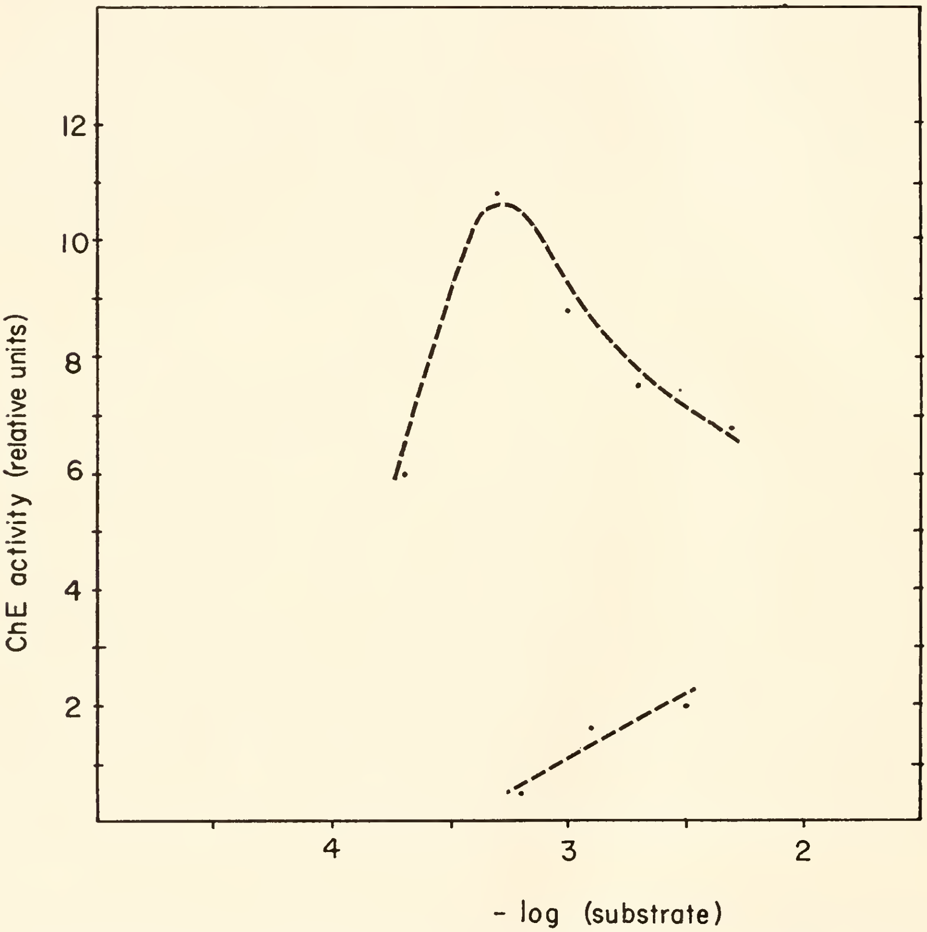

Figure 3. ChE activity as a function of PrThCh and BuThCh concentrations. The upper line describes activity for PrThCh; the lower line relates to BuThCh. The activities have been corrected for spontaneous hydrolysis of substrates. Enzyme activity is shown in relative units: one unit corresponds to a change in absorbance of 0.001 per minute.

TABLE I

ChE activity in brain homogenates of Cecropia

Source of brain	Number studied	Enzyme activity* (μmoles AThCh/brain-hr.)	Range	Remarks**
Larvae	4	0.24 ± 0.05	0.20–0.35	Late 5th instar, feeding
Prepupae	4	0.24 ± 0.02	0.22–0.28	Several hours before pupation
Fresh pupae	4	0.29 ± 0.03	0.24–0.33	2–22 hours after pupation
Unchilled pupae	6	0.32 ± 0.05	0.22–0.38	1–9 days after pupation
Unchilled pupae	8	0.33 ± 0.08	0.20–0.42	4–12 weeks after pupation
Chilled pupae	5	0.25 ± 0.03	0.22–0.31	8 weeks after pupation; then 10 weeks at 6° C.
Chilled pupae	8	0.58 ± 0.04	0.51–0.62	8 weeks after pupation; then 13–20 weeks at 6° C.
Developing adults	7	0.60 ± 0.05	0.51–0.69	2nd day of development

* Mean activity ± average deviation.

** Except where shown, all animals maintained at 25° C.

around the time of pupation, when the neurosecretory system becomes inactive, and when ChE was previously reported to undergo precipitous disappearance (Van der Kloot, 1955). It seems clear from Table I that except for possible minor fluctuations, an essentially unchanging level of ChE activity persists in the newly pupated animal, in unchilled pupae for at least 12 weeks after pupation, and in pupae chilled up to 10 weeks at 6° C. In several of the groups of unchilled pupae, one or two of the animals studied exhibited activity substantially higher or lower than the mean recorded in Table I, but no upward or downward trend in activity was noted during the indicated time intervals after pupation.

Table I also provides evidence for a rise in ChE activity during more prolonged chilling at 6° C. In each of eight pupae, examined at intervals after periods of chilling ranging from 13 to 20 weeks, the level of activity was at least double the mean activity recorded after ten weeks' chilling. Evidently the rise in activity occurred between the 10th and 13th weeks in this batch of animals. No trend of change in activity was noted between the 13th and 20th week. The elevated level was found to persist when chilled pupae were returned to 25° C. and allowed to initiate adult development (Table I). At all stages shown in Table I, the enzymatic activity was fully inhibited by eserine at 10^{-5} M. An AThCh concentration of 0.75 mM was found to be optimal in assays on larval and developing adult brains, as had earlier been established for pupae.

A total of 50 Cecropia pupae from the same batch as those just considered was returned to 25° C. after 16 weeks of chilling, and was used to provide animals at successive stages during the maturation of the adult moth after termination of diapause. As shown in Figure 4, ChE activity undergoes a six-fold increase during this period, when expressed on a "per brain" basis. In the first two weeks of adult development, activity rises progressively, but the high level thus attained persists without large change until the time of adult emergence. For unexplained reasons, a large variation in activity was observed in the later phases of adult development and in adults.

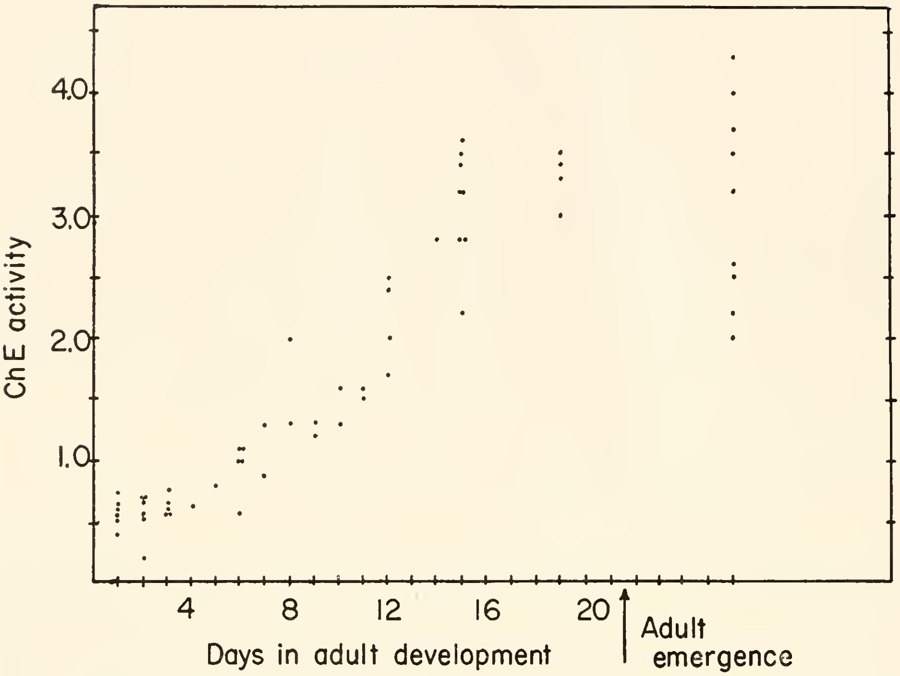

FIGURE 4. ChE activity in individual brains from developing adult and adult Cecropia. Each point represents the average of duplicate determinations on one brain. Enzyme activity is shown as micromoles AThCh per brain-hour. These units of activity are equivalent to those summarized in Table I.

4. *ChE activity in brains from various species of silkmoths*

The preceding assays were carried out on brains from the same batch of Michigan-reared Cecropia. To minimize the possibility that these data were exceptional, additional measurements of ChE activity were carried out, using several species of silkmoths. In more than three dozen additional unchilled and chilled Cecropia from different sources, ChE activity was encountered at approximately the same levels recorded in Table I. Activity was also detected in unchilled Cynthia pupae at about the same levels as in Table I. In unchilled Polyphemus pupae, the activity was somewhat lower, but still detectable. Measurements on Cynthia and Polyphemus were made using a final AThCh concentration of 0.75 m*M;* all activities were fully inhibited by eserine sulfate at 10^{-5} *M.* These observations provide additional evidence for essentially unchanging ChE activity in pupae stored at 25° C. or for at least two months at 6° C.

DISCUSSION

1. *Characteristics of ChE in silkmoth brain*

a. *Identification as AChE*

Throughout most of the life history, the enzymatic activity detected by histochemical and quantitative methods exhibits properties tentatively attributable to

AChE. This conclusion follows from the sensitivity displayed to certain inhibitors, from the reactivity toward thioester homologues, and from the substrate-activity curve obtained using AThCh. The Burroughs Wellcome agents, 62C47 and 284C51j, are strongly inhibitory and selective for vertebrate AChE's (Augustinsson, 1963), and also preferentially inhibit several ChE components of arthropod central nervous systems that have properties similar to vertebrate AChE's (Wigglesworth, 1958; Maynard, 1964). The lack of sensitivity to *iso*-OMPA reinforces this finding, since *iso*-OMPA is known to be relatively selective for vertebrate BuChE's including the pseudocholinesterase of mammalian brain (Aldridge, 1953; Austin and Berry, 1953; Pepler and Pearse, 1957). Further evidence for AChE in the case of Cecropia derives from the finding that activity is much lower toward BuThCh than toward AThCh. Finally, the pronounced reduction in activity at the higher concentrations of AThCh and PrThCh which we employed (Figs. 2 and 3) is typical of AChE's from vertebrate sources (Augustinsson, 1949, 1963). The combination of properties on the part of the Cecropia brain esterase is similar to that described for brain ChE in several insect groups (Gilmour, 1961). Insect ChE's possess properties that do not always lend themselves to convenient classification using vertebrate-based terminology (Chadwick, 1963). Thus, despite the similarities of Cecropia brain ChE to AChE, further study may reveal differences, and we use the term AChE with reservation.

In section 2.d of Results was mentioned the finding that ChE activity in adult brain includes a small but significant fraction which is sensitive to *iso*-OMPA at 10^{-4} *M*. This contrasts with the insensitivity to *iso*-OMPA at earlier stages in the life history, and suggests that an additional ChE component, perhaps with different specificity characteristics from AChE, may appear in the brain during the later phases of adult development.

b. *Changes in AChE during metamorphosis*

On the basis of the present study, the only major changes in AChE activity from the time of pupation through most of adult development are: (a) the two-fold rise in activity which occurs during chilling at 6° C.; and (b) the larger increase in activity that accompanies the growth and morphogenesis of the adult brain. With regard to the former, evidence is insufficient to determine whether the rise is attributable to fabrication of new elements within neuropile and concomitant synthesis of new ChE, or to enhanced titer of ChE within existing neuropile, or to other factors. In any event, the rise in activity during chilling appears to represent the earliest biochemical signal so far reported of the brain's change in neuroendocrine status, which occurs during storage at the low temperature (Williams, 1946, 1956). With regard to the increase of ChE during adult development, it seems most probable that the enzymatic changes in large part mirror the extensive morphogenetic events occurring at this time. A study of the behavior of brain esterases during adult development may yield useful information on changes at the cellular and subcellular levels underlying the physiological and morphological maturation of the adult brain.

c. *Localization of ChE in pupal brain*

In showing the presence of ChE in neuropile of pupal brain, our histochemical findings agree with those of Wigglesworth (1958) on the bug *Rhodnius prolixus,* and those of Salkeld (1961) on the milkweed bug, *Oncopeltus fasciatus.* In the former study, Wigglesworth concluded that staining in the neuropile, with AThCh as substrate, was largely confined to the glial elements. As used by us with Cecropia pupae, resolution of the histochemical method was insufficient to permit critical assessment of this important point. Wigglesworth also observed staining outside the neuropile when other esterase substrates were used, and his overall results supported the view that several esterases were present in the brain as a whole. Further information regarding multiple esterases and their potential significance will be presented in a subsequent section of this Discussion.

Wigglesworth (1958) noted in the first detailed histochemical study on localization of ChE in the brain of an insect that the restriction or near-restriction of AChE activity to neuropile shows certain kinship with the histochemical picture derived from studies on amphibians. Thus, in many regions of frog brain (*Rana pipiens*), heaviest staining due to AChE is found in areas rich in synaptic terminations and poor in cell bodies. However, in other areas of brain, neuron cell bodies also possess high ChE activity (Shen, Greenfield and Boell, 1955; Koelle, 1963). This situation in the frog differs markedly from that encountered in mammalian brains, as typified by rat or cat, in which AChE is abundant in neuron perikarya as well as in axonal and dendritic processes (Koelle, 1954; Pepler and Pearse, 1957). The localization of ChE in regions of synaptic contact has repeatedly prompted the suggestion that it is functionally involved in transmission; one can speculate similarly regarding the AChE which we detect in neuropile of Cecropia brain, but meaningful judgment on this point awaits further ultrastructural and physiological evidence.

2. *Analysis of present findings in relation to previous studies on Cecropia*

The results of the present study clearly show that substantial ChE activity persists in silkmoth brain throughout metamorphosis and diapause. Our findings therefore contrast with those of Van der Kloot (1955) in which ChE was not detected during diapause, but provide detailed evidence in favor of the conclusions suggested by Schoonhoven (1963) and Tyshtchenko and Mandelstam (1965) to the effect that ChE persists during the pupal diapause of lepidopterous insects.

Some of the discrepancy between our findings and those of Van der Kloot can be resolved if one considers certain properties of ChE in pupal brains as revealed in the course of the present study. It is worth noting that our spectrophotometric method has about the same sensitivity as the manometric technique used by Van der Kloot, since the minimum activity detectable by our method is close to that stated by Van der Kloot (0.05 micromoles substrate hydrolyzed per brain-hour). Although we have not compared the velocities of the ChE reaction in Cecropia with respect to AThCh and ACh, it would not be surprising if the rate were higher with AThCh, as this has been reported from time to time in the literature. Thus choice of substrate may have contributed to our success in detecting enzymatic activity during diapause. However, we believe that other factors are more significant, as stated below.

We find that ChE activity in homogenates of Cecropia brain declines by as much as 15% per hour in 100 m*M* phosphate buffer at pH 8.0 and 0° C., and more rapidly at 25° C. The activity also declines slowly when homogenates are stored in the frozen state at −15° C.; about 20% is lost after one week's time. The loss in activity would be significant in the case of manometric assays, which ordinarily require one-half to one hour, but much less significant in our spectrophotometric assays, which were completed within ten minutes. Thus, in the earlier study, it appears likely that much activity would have been lost during the 1–40-day periods of frozen storage prior to analysis, and during the manometric assays themselves.

There is ample reason to believe that our studies with AThCh yield information with regard to substrate preferences, substrate-activity relationships, and inhibitor sensitivities, that would also apply for ACh, as used in Van der Kloot's (1955) study. Our confidence derives from a number of studies in which the behavior of AThCh and ACh was compared, using the same ChE preparations (Heilbronn, 1959; Bergmann, Rimon and Segal, 1958; Ellman *et al.*, 1961). Thus we suspect that the 15 m*M* concentration of ACh used by Van der Kloot was supraoptimal for AChE and inhibitory in effect. This follows from our finding that the optimal AThCh concentration for pupal brain homogenates is only 0.75 m*M*, and that activity is markedly reduced at higher concentrations in the manner typical of AChE's (Fig. 2). Literature comparing substrate optima for AThCh and ACh (Heilbronn, 1959; Bergmann, Rimon and Segal, 1958; Ellman *et al.*, 1961), though based on vertebrate preparations, shows the optima to be much closer than the 20-fold difference separating our routine value for AThCh from that used by Van der Kloot (15 m*M*) for ACh. Moreover, in our experience the 0.5 *M* sodium chloride concentration incorporated in Van der Kloot's assay media yields a reaction rate lower than with buffer alone, when rates at substrate optima are compared. This conclusion concerning the effect of added salt conforms in large measure to that of Wolfe and Smallman (1956) on brain ChE from flies, with ACh as substrate. In the light of these arguments, and those of the preceding paragraph, we conclude that the potential activity of ChE in brains from diapausing pupae was not attained in earlier studies on Cecropia.

3. *Neurophysiological status of the brain during pupal diapause*

The present findings clearly show that at least one form of ChE persists in neuropile throughout diapause. It now becomes important to know its localization more precisely. In recent studies on the terminal abdominal ganglion of the cockroach, *Periplaneta americana,* Smith and Treherne (1965), using cytochemical techniques at the electron microscope level, have defined several sites of esterase activity. Eserine-sensitive esterase, presumably ChE, was found in association with axonal membranes at apparent synaptic sites in neuropile. Other sites of esterase activity were found both in and outside neuropile. If the neuropile-associated esterase which we detect in brains of diapausing Cecropia occurs at synaptic sites, and functions in transmission, then at least this element of neuronal interaction would appear to remain patent throughout pupal diapause.

In view of this possibility, and in the light of the still-uncertain electrophysiological status of the brain during diapause, a detailed reinvestigation by contemporary methods is clearly required. In studies of this type commenced recently, Walcott (personal communication, 1965) has confirmed certain electrical activity in the brains of diapausing silkmoth pupae; this supports the observations of Schoonhoven (1963) and argues that any electrical "silence" of the diapausing brain, as reported by Van der Kloot (1955) and Tyshtchenko and Mandelstam (1965), must be restricted to certain brain regions if it occurs at all. It also now becomes important to reassess the status of ACh itself following pupation, since this (assayed as cholinergic substance effective upon clam heart in bioassays) was reported to undergo precipitous disappearance at the outset of diapause, followed by more gradual reaccumulation in unchilled and chilled pupae (Van der Kloot, 1955).

4. *Multiple forms of esterase in relation to diapause and development*

The arguments in Section 2 of this Discussion do not explain our failure to detect large changes in esterase activity at the onset and termination of diapause, as reported by Van der Kloot (1955). It is possible that our findings are compatible with those of Van der Kloot, but reflect the behavior of different esterases. It has long been known that insect brains or heads contain a variety of esterases (see reviews by Gilmour, 1961; Chadwick, 1963), though few data are available on Lepidoptera. Recently, Maynard (1964) has characterized multiple esterases in the nervous systems of crayfish and lobster. In the light of these seemingly general attributes of arthropod central nervous systems, it is most probable that silkmoth brain likewise contains esterases beyond the single ChE detected in the present study. It is also worth noting that our use of AThCh at the rather low optimal concentration of 0.75 m*M* may preclude detection of esterases other than ChE's, or of ChE's having low activity toward the acetyl ester. Moreover, at higher concentrations, ACh may be hydrolyzed *via* enzymes other than ChE's; in Van der Kloot's study ACh was used at 15 m*M*. Thus the prospect merits attention that the enzymatic changes described in his study are meaningful, and apply to an esterase not detected in the present study. We look upon this prospect with favor, since exploratory spectrophotometric and electrophoretic studies on Cecropia brain, with various substrates, reveal several esterases including a component that undergoes changes at the onset and termination of diapause.

We therefore conjecture that an esterase other than the AChE described in this report, and perhaps already manifested in Van der Kloot's (1955) study, will be found to undergo changes that correlate with the neuroendocrine inactivation and reactivation of the brain. Such a correlation between enzymatic and physiological events would, of course, not in itself assure a causal role for the enzyme in the control of neurosecretion and diapause. Nonetheless, attention will surely center on its localization and properties in efforts to gain further insight into this control at the molecular and subcellular levels.

Meanwhile, the results of the present study clearly oblige us to abandon the attractive view that generalized disappearance and reappearance of ChE can account for neuroendocrine changes in the brain, that in turn bring about the onset and termination of pupal diapause. The history of biology is punctuated with occasions where investigators, confronted with the need to revise an earlier theory, have proceeded beyond their own data and sought to devalue the theory as a whole. Slater (1958) discusses instances of this type in the history of the study of cellular respiration. In present circumstances, we believe it prudent to continue to direct attention toward enzymatic events as part of the effort to understand the control of neurosecretion and diapause.

We gratefully acknowledge helpful discussions and technical advice from Drs. J. M. Allen, J. Martan, E. A. Maynard, and W. G. Van der Kloot. Dr. Charles Walcott generously permitted us to cite his unpublished electrophysiological observations. We also thank Burroughs Wellcome, Inc., for gifts of ChE inhibitors. R. S. Greathouse provided able technical assistance in a number of experiments.

SUMMARY

1. The localization and properties of cholinesterase in the brain of the Cecropia silkmoth were investigated by histochemical and quantitative spectrophotometric methods utilizing acetylthiocholine as substrate.

2. During pupal diapause, substantial activity was visualized in neuropile. At the outset of adult development, activity was also detected in adjacent regions occupied by neuronal or glial cell bodies.

3. Only one form of cholinesterase was detected with certainty. On the basis of substrate-activity relationships for acetylthiocholine, propionylthiocholine and butyrylthiocholine, and on the basis of its sensitivity to certain selective esterase inhibitors, the enzyme has properties of an acetylcholinesterase.

4. Substantial and essentially unchanging enzymatic activity was detected during pupation and most of pupal diapause, when the brain becomes endocrinologically inactive. However, an approximate doubling in activity was detected during storage of diapausing pupae at 6° C., apparently signalling the recovery of neuroendocrine competency by the brain. Subsequent growth and morphogenesis of adult brain were found to be accompanied by a six-fold further increase in activity.

5. Cholinesterase activity also persists during diapause in the Cynthia and Polyphemus silkmoths.

6. Consideration of the properties and optimal assay conditions for this enzyme in pupal brain assists in explaining previous reports that it was undetectable.

7. The presence of substantial cholinesterase activity throughout metamorphosis shows that a generalized disappearance and reappearance of the enzyme cannot be responsible for inactivation and reactivation of the neurosecretory mechanism that controls the onset and termination of diapause.

8. In the light of evidence for multiple forms of esterase in silkmoth brain, the present findings do not preclude a possible role for one or more esterases as part of the physiological mechanism controlling neurosecretion and diapause.

LITERATURE CITED

ALDRIDGE, W. N., 1953. Differentiation of true and pseudo cholinesterase by organophosphorus compounds. *Biochem. J.*, **53**: 62–67.

AUGUSTINSSON, K.-B., 1949. Substrate concentration and specificity of choline ester-splitting enzymes. *Arch. Biochem.*, **23**: 111–126.

AUGUSTINSSON, K.-B., 1963. Classification and comparative enzymology of the cholinesterases, and methods for their determination. *In:* Handbuch der Experimentellen Pharmakologie, Band 15, Cholinesterases and Anticholinesterase Agents (G. B. Koelle, sub-ed.), Springer-Verlag, Berlin.

Austin, L., and W. K. Berry, 1953. Two selective inhibitors of cholinesterase. *Biochem. J.*, **54**: 695–701.

Bergmann, F., S. Rimon and R. Segal, 1958. Effect of pH on the activity of eel esterase towards different substrates. *Biochem. J.*, **68**: 493–499.

Bullock, T. H., and G. A. Horridge, 1965. Structure and Function in the Nervous Systems of Invertebrates. W. H. Freeman and Co., San Francisco.

Chadwick, L. E., 1963. Actions on insects and other invertebrates. *In:* Handbuch der Experimentellen Pharmakologie, Band 15, Cholinesterases and Anticholinesterase Agents (G. B. Koelle, sub-ed.), Springer-Verlag, Berlin.

Chadwick, L. E., J. B. Lovell and V. E. Egner, 1953. The effect of various suspension media on the activity of cholinesterase from flies. *Biol. Bull.*, **104**: 323–333.

Ellman, G. L., K. D. Courtney, V. Andres, Jr. and R. M. Featherstone, 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.*, **7**: 88–95.

Ephrussi, B., and G. W. Beadle, 1936. A technique of transplantation for *Drosophila*. *Amer. Nat.*, **52**: 218–225.

Gilbert, L. I., 1964. Physiology of growth and development: endocrine aspects. *In:* The Physiology of Insecta (M. Rockstein, ed.). Academic Press, New York.

Gilmour, D., 1961. The Biochemistry of Insects. Academic Press, New York.

Gomori, G., 1952. Microscopic Histochemistry; Principles and Practice. University of Chicago Press, Chicago.

Heilbronn, E., 1959. Hydrolysis of carboxylic acid esters of thiocholine and its analogues. 3. Hydrolysis catalyzed by acetylcholine esterase and butyrylcholine esterase. *Acta Chem. Scand.*, **13**: 1547–1560.

Koelle, G. B., 1951. Elimination of diffusion artifacts in the histochemical localization of cholinesterases, and a survey of their cellular distributions. *J. Pharmacol. Exp. Therap.*, **103**: 153–171.

Koelle, G. B., 1954. The histochemical localization of cholinesterases in the central nervous system of the rat. *J. Comp. Neurol.*, **100**: 211–228.

Koelle, G. B., 1963. Cytological distributions and physiological functions of cholinesterases. *In:* Handbuch der Experimentellen Pharmakologie, Band 15, Cholinesterases and Anticholinesterase Agents (G. B. Koelle, sub-ed.), Springer-Verlag, Berlin.

Maynard, E. A., 1964. Esterases in crustacean nervous system. I. Electrophoretic studies in lobsters. *J. Exp. Zool.*, **157**: 251–266.

Pepler, W. J., and A. G. E. Pearse, 1957. The histochemistry of the esterases of rat brain, with special reference to those of the hypothalamic nuclei. *J. Neurochem.*, **1**: 193–202.

Salkeld, E. H. 1961. The distribution and identification of esterases in the developing embryo and young nymph of the large milkweed bug, *Oncopeltus fasciatus* (Dall.). *Canad. J. Zool.*, **39**: 589–595.

Schneiderman, H. A., and C. M. Williams, 1954. The physiology of insect diapause. IX. The cytochrome oxidase system in relation to the diapause and development of the Cecropia silkworm. *Biol. Bull.*, **106**: 238–252.

Schoonhoven, L. M., 1963. Spontaneous electrical activity in the brain of diapausing insects. *Science*, **141**: 173–174.

Shappirio, D. G., and C. M. Williams, 1957. The cytochrome system of the Cecropia silkworm. I. Spectroscopic studies of individual tissues. *Proc. Roy. Soc. London, Ser. B*, **147**: 218–232.

Shappirio, D. G., D. M. Eichenbaum and B. R. Locke, 1965. Cholinesterase in the brain of the Cecropia silkmoth in relation to the control of neurosecretion and diapause. *Amer. Zool.*, **5**: 698–699.

Shen, S. C., P. Greenfield and E. J. Boell, 1955. The distribution of cholinesterase in the frog brain. *J. Comp. Neurol.*, **102**: 717–743.

Slater, E. C., 1958. Catalytically active hemoproteins, with special reference to the cytochromes. *Biochemical Society Symposia*, **15**: 100–101.

Smith, D. S., and J. E. Treherne, 1965. The electron microscopic localization of cholinesterase activity in the central nervous system of an insect, *Periplaneta americana* L. *J. Cell Biol.*, **26**: 445–459.

Tyshtchenko, V. P., and J. E. Mandelstam, 1965. A study of spontaneous electrical activity and localization of cholinesterase in the nerve ganglia of *Antheraea pernyi* Guer. at different stages of metamorphosis and in pupal diapause. *J. Ins. Physiol.,* **11**: 1233–1239.

Van der Kloot, W. G., 1955. The control of neurosecretion and diapause by physiological changes in the brain of the Cecropia silkworm. *Biol. Bull.,* **109**: 276–294.

Wigglesworth, V. B., 1958. The distribution of esterase in the nervous system and other tissues of the insect *Rhodnius prolixus. Quart. J. Micr. Sci.,* **99**: 441–450.

Wigglesworth, V. B., 1964. The hormonal regulation of growth and reproduction in insects. *Advan. Ins. Physiol.,* **2**: 247–336.

Williams, C. M., 1946. Physiology of insect diapause: The role of the brain in the production and termination of pupal dormancy in the giant silkworm, *Platysamia cecropia. Biol. Bull.,* **90**: 234–243.

Williams, C. M., 1952. Physiology of insect diapause. IV. The brain and prothoracic glands as an endocrine system in the Cecropia silkworm. *Biol. Bull.,* **103**: 120–138.

Williams, C. M., 1956. Physiology of insect diapause. X. An endocrine mechanism for the influence of temperature on the diapausing pupa of the Cecropia silkworm. *Biol. Bull.,* **110**: 201–218.

Williams, C. M., and P. L. Adkisson, 1964. Physiology of insect diapause. XIV. An endocrine mechanism for the photoperiodic control of pupal diapause in the oak silkworm, *Antheraea pernyi. Biol. Bull.,* **127**: 511–525.

Wolfe, L. S., and B. N. Smallman, 1956. The properties of cholinesterase from insects. *J. Cell. Comp. Physiol.,* **48**: 215–225.

THE WATER ECONOMY OF SALAMANDERS: EXCHANGE OF WATER WITH THE SOIL

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Many species of amphibians spend their active periods at some distance from surface waters, and, consequently, cannot depend entirely upon ponds, pools, and streams for water to replace evaporative losses. These animals probably depend upon the water stored in the soil, and the availability of this water to them is an important parameter of their ecology.

Heatwole and Lim (1961) have shown that, at some water contents, the water in the soil was available to the salamander, *Plethodon cinereus*. They also showed that the lowest soil water level at which the salamander could withdraw moisture (the "absorption threshold") had the same moisture tension value in several soils. They thus introduced the moisture tension scale as a scale of the availability of soil water to salamanders.

In this study, I investigated the exchange of water between soil and salamanders in the soil, using six salamander species and a range of soil moistures. The following questions were investigated: (1) How does the rate of water exchange vary with the soil moisture? (2) What differences are there between different salamander species? (3) What variables other than the species and the soil moisture affect the exchange rates? (4) Can a salamander rehydrate fully in soil? With the data from these experiments, it is possible to predict the availability of water to these salamanders at any particular site.

Materials and Methods

The soil used was a homogeneous mixture of several local soils. Its characteristic curve, as determined on a porous plate apparatus (Soil Moisture Equipment Company; see Marshall, 1959, Ch. 2) is given in Figure 1. Lots of soil containing 10%, 6%, 4% and 1% water were packed into culture dishes. These lots provided a range of moistures from above field capacity (soil containg about 9% water) to air-dry soil (containing about 1% water).

The animals were dehydrated in air until they had lost approximately 15% of their initial body weight (this loss, expressed as a percentage of the initial weight, is the "dehydration deficit"). Each animal was then buried in a culture dish filled with soil at one of the four moisture contents. The animals were removed after six hours, and a soil sample was taken. Each salamander's bladder was emptied, and the salamander was then weighed to the nearest 5 mg. on a torsion balance (in the experiments with air-dry soils, the salamanders were not initially dehydrated, and were left in the soil only 2, 3 or 4 hours). The weight change over the six-hour interval was assumed to be due to water exchange, and was expressed as a rate [mg./(cm.^{2} × day)] where the surface area was calculated with Benedict's (1932) general formula:

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$$(\text{Surface Area}) = 10\ (\text{Body Weight})^{2/3}.$$

The soil sample was dried for 48 hours at 105° C. The soil moisture was calculated from the change in weight. Figure 2 shows the relation between soil moisture and exchange rate for this initial 6-hour period.

Some of the animals which were losing weight were returned to the soil and exposed for an additional 12- or 24-hour period. The weight changes over this period were used to determine the variation in water loss with the length of the exposure period.

All the animals which gained weight during the first 6-hour interval were returned to the soil. These salamanders were removed for weighing at 24-hour intervals until they had stopped gaining weight. The peak weight attained,

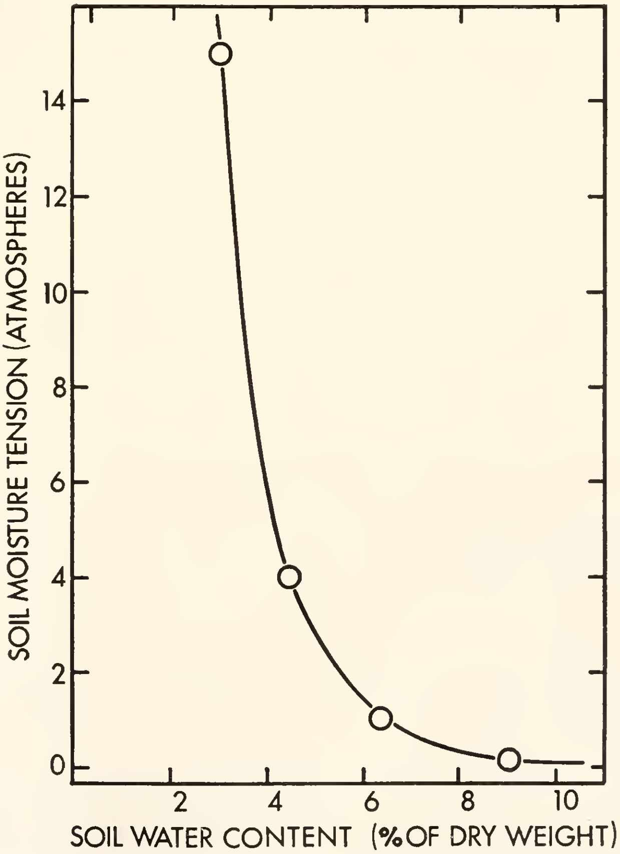

FIGURE 1. Characteristic curve of the soil used in the experiments. Each point is the mean of three values.

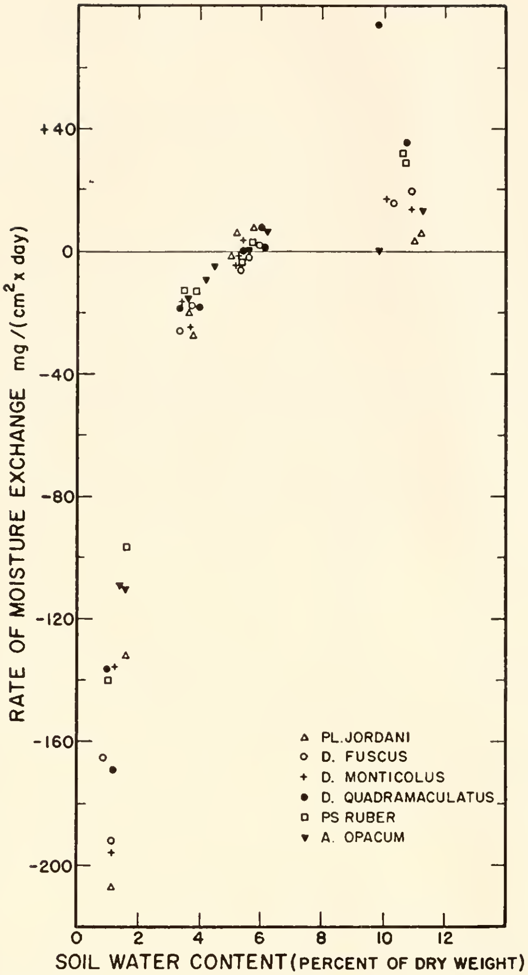

FIGURE 2. The relationship between soil water content and the rate of water exhange by six species of salamanders. The weights and dehydration deficits of the animals used in these experiments are given in Spight (1966a).

expressed as a percentage of the original weight, was the rehydration success of the animal.

All animals spent a final 48 hours in tap water. A weight increase during this period was assumed to indicate that the animals were unable to complete rehydration in soil.

Six species of salamanders were used in these experiments: the terrestrial, lunged *Ambystoma opacum,* and these lungless salamanders: *Desmognathus quadramaculatus,* from mountain streams; *D. monticolus,* from mountain stream banks; *D. fuscus,* from Piedmont streams and stream banks; *Plethodon jordani,* a forest floor form with no aquatic phase, and *Pseudotriton ruber,* a terrestrial form with an aquatic phase.

Results

Moisture tension and species as variables

The rate of water exchange with unsaturated soil is a function of the water content, and the function is the same for all of the species tested (Fig. 2).

The soil moisture tension is presumably a measure of water availability to salamanders, and, if Figure 1 is superimposed on Figure 2, it can be seen that the exchange rate is a function of the moisture tension. The moisture tension relationship will remain constant from soil to soil, and rates observed in these experiments, expressed as a function of the soil moisture tension, will serve as a basis for predicting performance in other soils. The water content allowing a particular exchange rate will, on the other hand, vary with the composition of the soil, and can be predicted for another soil only when the characteristic curve is available for that soil.

The range of moistures of critical importance to a salamander is the range about its absorption threshold, since it is only in soil above that threshold that a salamander can potentially remain in water balance. Within this range, the only variable which determines the rate and direction of water exchange over the six-hour measurement interval is the water content of the soil. The animals clustered about the 2 atm. (about 5%) point have a wide range of weights (1 g. to 10 g.) and dehydration deficits (8% to 20%) but all exchange water with the soil at rates solely dependent upon the water content of the soil (Fig. 2).

Additional variables in water exchange

Soil moisture was the only variable which affected the rate and direction of water flow between salamanders and soils when the soils had moisture tensions between 0.3 atm. and 15 atm. and when the measurement intervals were equal. In the saturated soils, however, the salamanders' rate of water uptake is correlated with both weight and dehydration deficit. The exchange rate by an individual salamander over a six-hour period in soil was also markedly different from that over a 12-hour period.

If the data from the two individuals of each species which rehydrated in saturated soil are compared, the differences between the members of each pair are correlated with differences in body weight and dehydration deficit. For examples, the larger of the pair of *D. fuscus* had the lower rate of gain, both animals having the same dehydration deficit. For the pairs of *D. monticolus* and *Plethodon jordani* of the same weight, the animal in each pair with the greatest dehydration deficit had the greatest rate of uptake. For pairs of salamanders of the species *Pseudotriton ruber* and *A. opacum,* the smaller animal had the greater deficit and absorbed water from the soil more quickly. The data for *D. quadramaculatus* are ambiguous. In all cases, for animals with similar dehydration deficits, the rates of rehydration in soil were well below the average rates of rehydration in water [rates in water ranged from 24 mg./(cm.^{2} × day) to 190 mg./cm.^{2} × day); see Spight, 1966b].

The rate of loss by salamanders to air-dry soil (by the 12 animals with the highest loss rates in Figure 2) is inversely correlated with the time the animals spent in the soil. The four animals which spent only two hours in soil had an average rate of loss of 190 mg./(cm.^{2} × day); all their rates fall within the range of rates of loss by animals which were dehydrated in room air [for 60% to 70% relative humidity at 20° C., animals lost 100 to 310 mg./(cm.^{2} × day) by evaporation to the air; see Spight, 1966a]. In contrast, animals which spent 3 hours or 4 hours in soils of the same water content averaged, respectively, 140 and 110 mg./(cm.^{2} × day).

The mediating influence of the soil is particularly prominent in the air-dry soils, for in these soils a small amount of water lost by a salamander to the surrounding soil will lower the tension of that soil markedly (note the slope of the characteristic curve in Figure 1 for soils containing less than 4% water). The mediation was also evident in the moist soils. When one individual spent successive intervals of 6 and 12 hours in the same soil, the loss rate was appreciably lower over the longer interval (Table I).

TABLE I

Rates of water loss by individuals to moist soils during successive periods of different lengths in the same soil

Species	Rate of water loss		Soil moisture %
	Hours 1 to 6 mg./(cm.^{2} × day)	Hours 6 to 18 (mg./(cm.^{2} × day)	
Desmognathus fuscus	−18.2	−13.8	3.71
D. monticolus	−16.4	−11.4	3.46
D. monticolus	−25.0	− 9.4	3.70
D. quadramaculatus	−18.6	−11.4	3.42
D. quadramaculatus	−18.5	− 8.9	4.00
Plethodon jordani	−19.9	−10.0	3.64
Plethodon jordani	−27.5	−10.5	3.78
Ambystoma opacum	−16.6	−13.0	3.62
Pseudotriton ruber	−12.9	− 8.3	3.58
Pseudotriton ruber	−13.1	− 8.6	3.91

Rehydration ability

The salamanders can complete rehydration in saturated soil, although the rate of gain is slow in comparison with rates of rehydration in water. Twelve animals attained 93.2% of their initial weights (SE = 0.6) from soils with 9% to 11% water, taking one to four days to complete the rehydration. These animals made no further weight gains during 48 hours in water.

Animals exposed to soils containing 5% to 6% water showed initial weight gains, but these soils dried below the absorption threshold before the animals were able to complete rehydration. These animals subsequently attained 92.4% of their original weights by rehydration in water (SE = 1.1, N = 12); this percentage is not significantly different from the percentage attained by the group of salamanders which were able to complete rehydration in soil ($P > 0.50$ that the difference has arisen by chance alone).

It is postulated that a salamander can complete rehydration in any soil from which it can gain any water, although it is doubtful that this can be shown in the laboratory, since it is extremely difficult to maintain soil at a precise moisture tension.

Discussion

Salamanders are found in both "wet" and "dry" communities, and species can be characterized by their "water requirements." Some species are wholly aquatic, others semi-terrestrial, and others wholly terrestrial. Investigators, including G. K. Noble (1931), have suggested that the distributions of different amphibian species might be correlated with specific differences in their ability to absorb water from various substrates. This study has shown that if the soil water at a particular site is available to a terrestrial species, it will also be available to a characteristically aquatic species. In other words, in spite of well documented differences in "water requirements," knowledge of the availability of water in the substrate of a habitat cannot be used to make predictions about which salamander species will be able to occupy that habitat. Salamanders thus present another example of Beament's (1961) generalization that, among closely related animals, even species from quite different habitats have only minor differences in their physiologies.

Salamanders can absorb water from soils with moisture tensions less than 2 atm. The meaning of this value in typical natural situations can be clarified by pointing out three particular points on the moisture tension scale. Soil is normally considered (1) to be at a tension of 0.33 atm. when the gravitationally-induced draining of a rain-wet soil is complete, and (2) to be at a tension of 15 atm. when a plant growing in the soil becomes permanently wilted. Van Bavel (1953) gives the third point (3): on non-irrigated crop land near Raleigh, N. C., during the average year there will be 20 days during which the soil moisture rises above 0.77 atm. (he considers this tension to be a stress level for agricultural plants). It may be seen, then, that salamanders can obtain water from agricultural land in North Carolina throughout most of the year.

Soil properties and moisture exchange

As the time the salamander spends in soil below the absorption threshold increases, the rate of water loss by the salamander to the soil drops. This rate drop is more prominent in the air-dry soils, but it is also evident in the wetter soils. These rate drops reflect the water conductance of the soil used, and a related phenomenon, the wetting front.

In unsaturated soils (soils with tensions greater than 0.33 atm.), a wetting front, a locally steep gradient of moisture content, is formed between a wet region and a drier region (Klute, 1952). The gradient is such that in very dry soil there may be no movement of liquid water from a wet region (at about field capacity) to an adjacent, very dry region (air dry; Bodman and Colman, 1944).

As water is lost by the salamander to the soil, it is absorbed by the immediately adjacent soil, and a wetting front is formed. The water lost by the salamander accumulates between the salamander and the edge of the wetting front. In this zone, the humidity increases with the accumulation of water; thus the accumulated water lowers the humidity gradient from the salamander to the adjacent soil, and the evaporation rate of the salamander decreases.

Soil water at some moisture tensions is available to many salamander species, and the availability of the soil water at a particular site to a salamander can be predicted. With this information, it is possible to approach old problems, such as "does water act as a limiting factor in the distribution of salamanders," and "what are the water problems of hibernating salamanders?" These experiments have not shown that anurans have the same absorption threshold as do these urodeles. Such a demonstration would make an interesting future study, and would lead directly to the solution of the problem, "where do desert toads get their water?"

I would like to express my gratitude to Dr. Paul J. Kramer and Dr. Peter J. Bentley for advice and encouragement during the course of this study. I am indebted to Nancy Nickerson for many of the animals, to Dave Hillier for the moisture tension curve, and to Lee Miller for the soil. Dr. Bentley and Dr. Peter W. Hochachka were kind enough to read the manuscript. Dr. J. R. Bailey supervised the work and identified the animals.

Summary

Specimens of six salamander species were exposed to different soil moistures, and rates of water exchange were calculated from the changes in weight observed. Soil water was available to these salamanders in soils with moisture tensions between 0 atm. and 2 atm. At these tensions, salamanders could rehydrate fully, and can therefore be expected to remain in water balance in any soil with a tension in this range. The measurements should be useful in determining the suitability of habitats for salamanders. The rate of water exchange between the salamander and the soil was a function of the soil moisture tension. The rate of uptake from saturated soils was correlated with the body weight and the dehydration deficit of the salamanders. There were no differences in rate of exchange or in absorption threshold between the different species. These characteristics of water exchange between salamanders and soil are related to the properties of the soil.

LITERATURE CITED

Beament, J. W. L., 1961. The role of physiology in adaptation and competition between animals. *Symp. Soc. Exp. Biol.*, **15**: 62–71.

Benedict, F. G., 1932. The physiology of large reptiles. Carnegie Inst. Washington Publ. 425.

Bodman, G. B., and E. A. Colman, 1944. Moisture and energy considerations during downward entry of water into soils. *Soil Sci. Soc. Amer. Proc.*, **8**: 116–122.

Heatwole, H., and K. Lim, 1961. Relation of substrate moisture to absorption and loss of water by the salamander *Plethodon cinereus*. *Ecology*, **42**: 814–819.

Klute, A., 1952. A numerical method for solving the flow equation for water in unsaturated materials. *Soil Science*, **73**: 105–116.

Marshall, T. J., 1959. Relations between water and soil. Commonwealth Bureau of Soils, Harpenden, England. Technical Communication 50.

Noble, G. K., 1931. The Biology of the Amphibia. McGraw Hill, N. Y.

Spight, T. M., 1966a. Studies on the water relations of salamanders. M.A. thesis, Duke University.

Spight, T. M., 1966b. The water economy of salamanders: water uptake after dehydration. *Comp. Biochem. Physiol.* (in press).

Van Bavel, C. H. M., 1953. A drought criterion and its application in evaluating drought incidence and hazard. *Agronomy J.*, **45**: 167–172.

STUDIES ON THE TREMATODE GENUS PARAMONOSTOMUM LÜHE, 1909 (DIGENEA: NOTOCOTYLIDAE)[1]

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The genus *Paramonostomum* was erected by Lühe (1909) with *Monostoma alveatum* Mehlis in Creplin, 1846 (syn. *Monostoma alveiforme* Cohn, 1904) as type. The species had been included by Monticelli (1892) in the genus *Notocotylus* Diesing, 1839, but Lühe predicated that it is not congeneric with *Notocotylus triserialis* Diesing, 1839, type of *Notocotylus*. The species, *P. alveatum,* has been reported from a large number of birds including *Anas* spp., *Anser anser, Nyroca marilla, Oedemia* spp., *Somateria mollissima, Cygnus* spp., *Branta* spp., and *Clangula hyemalis.* Some 20 additional species of *Paramonostomum* have been described but distinctions between certain of them are very tenuous.

One life-cycle, that of *P. alveatum,* was reported by Kulachkova (1954). The work was done at the marine station on Kandalaska Bay, in the southwest portion of the White Sea, longitude 33° East and latitude 65.5° North. *Hydrobia ulvae* was the intermediate host and harbored the asexual generations of the parasite. Mme. Kulachkova published two short papers (1961a, 1961b) on seasonal infection of the mollusks and on the biology of the larval stages of *P. alveatum.* I am indebted to Dr. Galtsoff, who graciously translated the Russian texts for me. The studies of Mme. Kulachkova were occasioned by the mass mortality of young eider ducks; in the period 22 June to 7 July, 1949, 321 chicks died from the infection. As many as 50,000 worms were found in a single bird. The parasites penetrated between the intestinal villi, with inflammation and destruction of the epithelium and membranes. Fourteen per cent of the *H. ulvae* in the tide-pools were infected and the cercariae, on emergence, encysted promptly on the shells of the snails from which they had emerged. The cysts were 0.155 mm. in diameter and the worms matured in 6–8 days in the birds. The tide-pools had sandy-gravelly bottoms and the eider chicks, less than two weeks old, fed in these tide-pools where the shells of the hydrobias carried from 10 to 25 cysts per snail. Birds older than two weeks, fed in *Fucus* and mussel beds where the hydrobias were rare or absent, and birds older than two weeks survived. In the examination of 5427 snails over a four-year period, the rate of infection varied from 3.3% to 12%, with the greatest incidence in July and August. Shedding of the larvae began at water temperature of 23° and massive discharge in the range 23° to 26°. This was usually in the last week of June and first 10 days of July, although the dates varied with weather conditions, but this was the time when the eider chicks were feeding in the tide-pools.

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[1] Investigation supported by NSF, GB-3606, continuation of G-23561.

Miriam Rothschild (1938) had studied the cercariae of the notocotylid trematodes and described six species of these cercariae from the snails, *Peringia ulvae* and *Hydrobia ventrosa*. They were assigned to three different groups, based on the form of the excretory system: the MONOSTOMI group, in which the anterior transverse portion of the vesicle is a closed tubular circuit situated posterior to the median eye-spot and cerebral ganglion; the IMBRICATA group, in which the anterior portion of the vesicle forms a loop, between the eye-spots, which passes anterior to the median eye-spot and cerebral ganglion; and the YENCHINGENSIS group, in which there is an unpaired finger-like diverticulum which extends anteriad from the transverse portion of the vesicle.

Stunkard (1965) reported on the examination of more than 4000 specimens of *Hydrobia salsa* (Pilsbry, 1905) taken from brackish-water ponds, near Woods Hole, Massachusetts, and the discovery of at least four species of notocotylid cercariae, including the larval stages of *Paramonostomum alveatum*. The study has been continued and a fifth notocotylid species has been identified (Stunkard, 1966a). Two species have Yenchingensis type excretory systems; the metacercariae develop in the intestinal caeca of chicks and of domestic and eider ducklings, and belong to the genus *Notocotylus*. One of these species is identical with *Notocotylus minutus* Stunkard, 1960 and the other is described as a new species (Stunkard, 1966b). One of the five cercariae belongs to the Imbricata Group; it develops in the bursa Fabricius of chicks and domestic ducklings and may be identical with *Uniserialis gippyensis* Burton, 1958. The two other species have *Monostomi* type excretory systems; the metacercariae develop in the intestine of chicks and both domestic and eider ducklings, and belong to the genus *Paramonostomum*. One is identified as *P. alveatum;* and the other is *Paramonostomum parvum* Stunkard and Dunihue, 1931.

The methods for study of the *Paramonostomum* species are identical with those described for the species of *Notocotylus* (Stunkard, 1966b). The procedure is relatively simple. The infected snails were discovered by isolation; the larvae were studied alive, with and without the use of vital dyes. All stages were fixed and stained for subsequent study. Duboscq-Brasil and AFAG mixtures were used for fixation and specimens prepared as whole mounts were stained with Mayer's paracarmine, Semichon's acetic carmine, or Ehrlich's acid haematoxylin. Adult worms, sectioned serially in transverse and frontal planes, were stained with haematoxylin and erythrosin. Snails were isolated singly and metacercariae, encysted on the light side of the container, were fed at two-day intervals to rats, mice, hamsters, young herring gulls, chicks and both domestic and eider ducklings. The eider chicks were provided through the kindness of Mr. Walter Welch and his associates on the staff of the U. S. Fish and Wildlife Service, Boothbay Harbor, Maine. The snails were killed to identify the larval stages and the final hosts were killed to recover the developing and sexually mature worms. No infection was obtained in a mammalian species. Both species of *Paramonostomum* developed to maturity in chicks and in both eider and domestic ducklings, but sexually mature worms were not recovered from gulls. The intestine of a gull killed two days after the ingestion of about 200 metacercariae contained a few dead, excysted worms. Three live, juvenile specimens, identified as *P. parvum,* were recovered from the intestine of another gull fed metacercariae on 29 June, 1964, and autopsied 22 July, 1964.

The worms were the same size; fixed and stained they are 0.33 mm. long and 0.18 mm. wide, with very large ovaries and testes, but without eggs in their uteri. The two species of *Paramonostomum* are distinguished primarily by differences in size and size of organs. There are constant and apparently significant differences in sizes of cercariae, metacercariae and sexually mature adults. Twelve specimens of *P. alveatum* were left August 30, 1966, for several hours in pond-water and a large number of eggs were expelled by the worms. Three days later six young laboratory-reared *H. salsa* were placed in the dish with the eggs and were observed to eat some of them. The snails were then removed to fresh pond-water and a snail sacrified on October 3, 1966, contained rediae, some of which contained developing cercariae. The experimental infection of laboratory-reared *H. salsa* completes the life-cycle and confirms the earlier observations. The findings of the present study confirm the account of Mme. Kulachkova on *P. alveatum*.

Descriptions

Paramonostomum alveatum (Mehlis in Creplin, 1846)

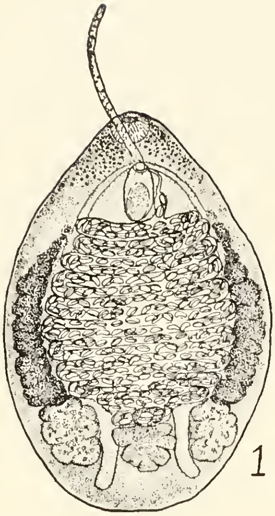
(Figs. 4–6)

Previous accounts include the inadequate redescription of the original specimens by Monticelli (1892), the brief statement by Lühe (1909) when he erected the genus *Paramonostomum*, and the account by Kossack (1911).

Adult (Fig. 4)

The worms are ovate, rounded posteriorly, more pointed anteriorly. Typically, the edges of the body are turned ventrad and mediad, so the venter forms a cupuliform cavity, which suggested the specific name, *alveatum*. The opening is smaller than the outline of the body. Fixed and stained sexually mature specimens measure 0.50 to 0.85 mm. in length and 0.40 to 0.53 mm. in greatest width, which is in the posterior half of the body. Under pressure of a coverglass, the dimensions of living worms are much greater. The cuticula appears to be smooth, but examination of living specimens under high magnification discloses exceedingly minute, closely set spines, arranged in parallel rows, on the ventral surface. The musculature of the body wall is weak and movement is slight or sluggish. The pigment from the ocelli of the cercaria persists in the parenchyma of the anterior end of the body. The oral sucker is 0.06 to 0.065 mm. in diameter; the esophagus is short, about the length of the oral sucker; the caeca are dorsal in location and follow the lateral contours of the body; they turn mediad at the anterior ends of the testes, pass between the testes and ovary, and terminate blindly behind the level of the gonads. As noted by Rothschild (1941, p. 363, fig. 1), "it is well known that in mature notocotylid trematodes the excretory vesicle becomes greatly complicated." Essentially, large, ramifying dendritic branches arise from the lateral and medial sides of the ring formed by the fusion of the collecting ducts of the cercaria, and constitute a complex reticulum that permeates the parenchyma of the body. The bladder, situated posterior to the ring, opens to the surface by a dorsal pore near the caudal end of the body.

The testes are oval, lobed organs, situated in the extracaecal areas at the poste-



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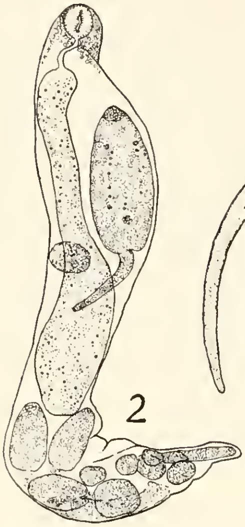

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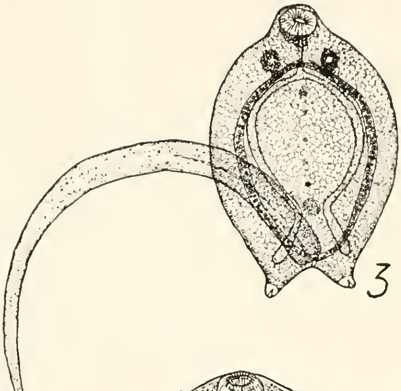

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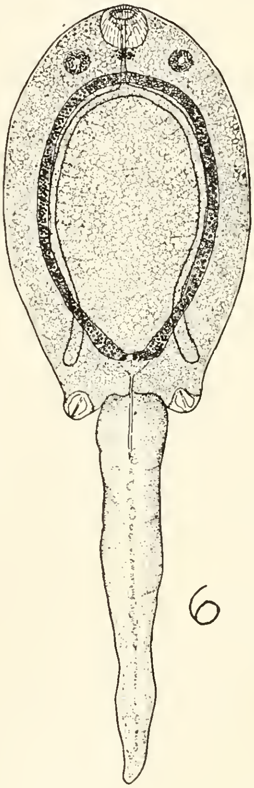

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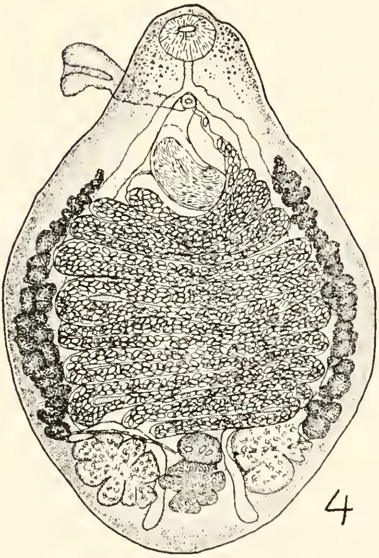

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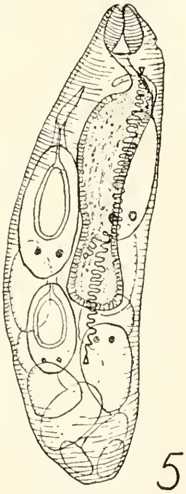

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FIGURE 1. *Paramonostomum parvum,* adult specimen, ventral view, from eider duck; worm, 0.37 mm. long.

FIGURE 2. *P. parvum,* redia, pressed specimen, 0.93 mm. long.

FIGURE 3. *P. parvum,* cercaria, ventral aspect, fixed without pressure in hot whirling solution (Duboscq-Brasil), body, 0.17 mm. long.

FIGURE 4. *P. alveatum,* adult specimen, ventral view, from eider duck; worm, 0.75 mm. long.

FIGURE 5. *P. alveatum,* redia, fixed without pressure, 0.82 mm. long.

FIGURE 6. *P. alveatum,* cercaria, ventral aspect, fixed under pressure of coverglass, body 0.32 mm. long.

rior end of the body. They measure 0.12 to 0.18 by 0.10 to 0.14 mm. in diameter. Sperm-ducts arise at the anteromedian faces and unite in front of Mehlis' gland to form the vas deferens which passes anteriad, dorsal to the uterine coils. About one-third of the body-length from the anterior end, the duct enlarges to form a large, coiled, external seminal vesicle, the last loop of which extends forward on the right side of the cirrus sac. The cirrus sac is curved, short and broad, 0.16 to 0.24 mm. long and 0.10 to 0.13 mm. wide; the posterior portion contains the large internal seminal vesicle; the pars prostatica is short, and the cirrus is eversible (Fig. 4). The ovary is median, between the testes. It is a variably lobed organ, 0.08 to 0.15 mm. in diameter. The oviduct arises at the anterodorsal face of the ovary and receives a short, common vitelline duct as it enters Mehlis' gland, which is smaller and immediately anterior to the ovary. Mehlis' gland contains the ootype, in which the eggs are formed. The initial coils of the uterus are filled with spermatozoa. There are 10 to 12 transverse uterine loops that occupy the intercecal area posterior to the cirrus sac. They are ventral to the digestive caeca and in pressed specimens the vitellaria are almost contiguous with the uterine loops. The metraterm is ventral to the cirrus sac and about one-half its length. The vitellaria are extracaecal and extend from the testes to the level of the cirrus sac. The eggs are operculate, have long polar filaments, measure 0.019 to 0.021 by 0.011 to 0.012 mm., and are embryonated when passed.

Redia (Fig. 5)

The rediae are vermiform, cylindrical to sausage-shaped, and vary in size from young, colorless individuals with small germ balls to large, gravid rediae that may extend to a length of 1.20 mm., and when retracted may be 0.50 mm. in width. The size of the redia is largely determined by the number and size of the progeny in it. The large rediae are golden-yellow to orange in color. The body has annular and longitudinal muscles and when the longitudinal muscles are contracted, the wall has a crenate margin. The pharynx increases to 0.055 mm. in diameter and the esophagus is about the same length. In young rediae the intestine may extend more than one-half the length of the body, but it becomes relatively shorter as the redia is filled with offspring. The intestine is filled with decomposing snail tissue, yellow droplets and blackish amorphous material. The excretory pores are lateral, in the posterior half of the body. From each pore a common duct passes forward for a short distance and divides into anterior and posterior branches, each of which terminates in a flame-cell. The collecting ducts are coiled and are enclosed in straight-walled sinuses as described by Rothschild (1935).

Cercaria (Fig. 6)

The cercariae leave the rediae before they are mature and complete their development in the haemal sinuses of the snail. They emerge from the snails principally between 10 AM and 2 PM, and swim ordinarily for one to three or four hours. They encyst on the shell or operculum of the snail from which they emerged or on any hard surface. When irritated, *e.g.*, placed in a solution of vital dyes, they may encyst at once. When swimming, the body is contracted, bent ventrally; the tail is extended and lashes violently. They are photosensitive and accumulate on the light side of the container. The body is oval to ovate, more pointed anteriorly, convex dorsally and concave ventrally; contracted it is circular, 0.20 to 0.25 mm. in diameter; elongated it may be 0.38 by 0.15 mm. The tail is simple, 0.20 to 0.50 mm. long and when extended it is 0.02 to 0.03 mm. wide at the base. When the body is extended the tail is contracted and *vice versa.* The posterolateral ends of the body bear eversible and retractile locomotor appendages, 0.020 to 0.025 mm. in diameter; when the body is extended they are close together, separated only by the base of the tail which is ventral to them; when the body is retracted they are at the posterior, dorsolateral corners. While in the redia, the cercariae have only two ocelli, with scattered pigment around the eye-spots, but by the time of emergence often there is a third, median, ring-like condensation of pigment between the ocelli, and dendritic strands of pigment extend posteriad along the digestive caeca. The ocelli are 0.018 to 0.020 mm. in diameter; they are provided with lenses and are situated dorsal and anterior to the ganglia of the nervous system. The parenchyma becomes filled with unicellular cystogenous glands; the secretion is in the form of bacilliform rods, 0.003 to 0.004 mm. in length and about one-half as wide. The oral sucker is 0.038 to 0.045 mm. in diameter; the esophagus is about the same length and crosses the excretory ring dorsally, whereupon it opens into the caeca. The caeca are dorsal and medial to the excretory ring as they pass posteriad, but near the posterior end of the body they turn laterad across the ring and end blindly as shown in the figure. The development of the excretory system of the cercaria agrees completely with the account of Rothschild (1935) on *C. ephemera* Lebour, 1907. In young cercariae the excretory pores are on the sides, near the middle of the tail, but in mature larvae, the portion of the system in the tail atrophies and a new excretory pore develops from the dorsal wall of the excretory bladder. The ring, formed by the coalescence posteriorly and anteriorly of the collecting ducts, is filled with concretions. In the posterior part of the ring there may be four to six concretions at any level, while in the anterior part of the ring the concretions may be disposed in a single row. They measure 0.003 to 0.006 mm. in diameter and often two or three are fused.

Metacercaria

In encysting, the cercaria attaches by the oral sucker; the body is contracted to circular form, and the cystogenous material is extruded on all sides. As the secretion hardens, the tail, which is left outside, lashes itself free and the cyst, 0.15 to 0.16 mm. in diameter, is firmly attached to the substratum. The cyst wall is relatively impermeable and resists desiccation; the larva moves in the cyst and if not dried, remains infective for a long period, weeks, possibly months.

Paramonostomum parvum Stunkard and Dunihue, 1931
(Figs. 1–3)

This species, described originally from specimens found in the intestine of an unidentified duck, was recovered after feeding metacercariae to laboratory-reared eider and domestic ducklings and to day-old chicks. The asexual generations were found in *Hydrobia salsa* taken from Nobska and Oyster Ponds, brackish-water areas that communicate with Vineyard Sound, near Woods Hole, Massachusetts.

The specimens of experimental infection agree completely with the description of worms of natural infection as reported by Stunkard and Dunihue (1931).

Adult (Fig. 1)

The worms measure 0.25 to 0.50 mm. in length and 0.20 to 0.35 mm. in width. Only much flattened specimens exceed 0.50 mm. in length. In the original report, the presence of spines on the cuticula was regarded as doubtful. With an abundance of material, it has been possible to observe the presence of exceedingly minute, closely-set spines on the ventral surface of the body. The spines are arranged in fine, parallel rows. They are not visible on fixed and stained specimens, but on living worms under high magnification they can be resolved by careful focussing.

Redia (Fig. 2)

The rediae closely resemble those of *P. alveatum;* they occupy the haemal sinuses of the snail and grow to a size of 0.65 by 0.13 mm. or 0.75 by 0.10 mm. Daughter rediae emerge from the parental rediae while very small, much smaller than the cercariae when they emerge, and while small they are active and migratory. As the body cavity becomes filled with germ-balls and developing cercariae, the rediae become more sluggish. The pharynx increases to a diameter of 0.035–0.042 mm., and when pressed may measure 0.05 mm. in diameter. The esophagus is approximately as long as the pharynx and the caecum varies with the size of the redia. In young specimens the caecum is long, often more than one-half the body length, but is relatively shorter as the redia enlarges. The caecum is filled with partially digested snail tissue, yellow droplets and amorphous, blackish material. The young rediae are colorless but as they grow they become more and more filled with orange-yellow material. The excretory system is identical with that of the redia of *P. alveatum.*

Cercaria (Fig. 3)

The cercariae differ from those of *P. alveatum* principally in size. They emerge at about the same time of day, have the same swimming movements, accumulate on the light side of the container, and encyst on the shells of the snails or other hard surface in the course of one to three or four hours. They emerge from the rediae while still immature and at this stage have considerable dark pigment around the ocelli and in the anterior third of the body. On emergence from the snail, the body is oval to ovate, more pointed anteriorly, convex dorsally and concave ventrally; contracted it is circular, 0.14 to 0.16 mm. in diameter; extended it may be 0.30 by 0.10 mm. The tail is simple, slender; it varies from one-half to three times the length of the body. The locomotor appendage pits at the posterolateral ends of the body are smaller than those of *P. alveatum* and diverge at an angle in specimens killed without pressure in whirling, hot, fixing fluids (Fig. 3). The ocelli are dorsal and anterior to the cephalic ganglia and measure about 0.015 mm. in diameter; they are provided with lenses. The oral sucker is 0.029 to 0.036 mm. in diameter; the esophagus is approximately the same length; the caeca follow the lateral contours of the body until they turn laterad and cross the excretory ring dorsally, near the posterior end of the body. The body is filled with cystogenous cells; the secretion appears as bacilliform rods, 0.002 to 0.003 mm. long and about one-half as wide. There are 12 to 15 cells between the caeca in a transverse section through the middle of the body. The excretory system develops as in all notocotylid cercariae; the ring passes posterior to the ganglia and ocelli and is filled with concretions; they vary from 0.003 to 0.006 mm. in diameter. On either side, a recurrent tubule passes posteriad from the anterolateral faces of the ring; the recurrent tubule bears tufts of long cilia and near the middle of the body divides into anterior and posterior branches. Each branch bears three clusters of flame-cells, probably three in each cluster, but not all cells have been observed, as the cystogenous cells begin to fill with secretions before all the cells and tubules of the midbody are recognizable.

Metacercaria

The cercariae encyst promptly if irritated by agitation of the water or the presence of toxic substances, *e.g.*, solutions of vital dyes; otherwise they may swim for one to three or four hours. They encyst on the operculum or shell of the snail or any hard surface, including the wall of the container, always on the side toward the light. The cysts measure 0.13 to 0.14 mm. outside diameter and 0.11 to 0.12 mm. inside diameter. A specimen was fixed while encysting on a slide; the secretion had produced a thin, flexible membrane and outside the membrane there was a sheet of seta-like projections of cystogenous material, 0.030 mm. long and 0.003 mm. in diameter.

Discussion

Rothschild (1941) reported efforts, continued for five years, to solve the life-histories of the notocotylid cercariae that parasitize *Peringia ulvae*. Six species of larvae were isolated and cysts were fed to laboratory-reared ducklings. Three of the species belonged to the Monostomi group and three to the Yenchingensis group of cercariae. All attempts to obtain adult worms from the Monostomi cercariae were negative but she reported (p. 363), "Two species of the Yenchingensis sub-group, however, developed in the intestinal ceca of the ducks, into flukes of the genus *Paramonostomum*." Rothschild noted with some surprise that one cercaria of the Yenchingensis group (Szidat and Szidat, 1933) and one of the Monostomi group (Yamaguti, 1938) had been reported to develop into the same species of adult, *Notocotylus attenuatus*. Although Rothschild obtained adult specimens referred to the genus *Paramonostomum*, the specific identity of the specimens was not determined. Kulachkova (1954) did not assign the cercariae of *P. alveatum* to one of the larval groups. The recent studies of Odening (1966) are particularly interesting; he reported that the cercariae of *Notocotylus ephemera* (Nitzsch, 1807); *Notocotylus noyeri* Joyeux, 1922; *Notocotylus pacifer* (Noble, 1933); *Notocotylus ralli* Baylis, 1936; and *Notocotylus regis* Harwood, 1939, all of which develop in fresh-water, pulmonate snails, belong to the Monostomi group of cercariae; whereas the cercariae of *Catatropis verrucosa,* which also develop in fresh-water pulmonates, *Segmentina nitida* and *Gyraulus albus,* belong to the Imbricata group. From the studies of Stunkard (1960) on the life history of

Notocotylus minutus and (1966b) on *Notocotylus atlanticus,* it appeared that cercariae of species of *Notocotylus* belong to the Yenchingensis group and mature in the digestive caeca of birds, whereas the cercariae of *Paramonostomum* belong to the Monostomi group and develop in the lumen of the intestine. However, the statements of Rothschild and Odening do not permit such a correlation between larval type, developmental site, and generic allocation, and the significance of the larval groups remains obscure and equivocal.

The genus *Paramonostomum* contains some 20 described species, but the distinctions between certain of them are very tenuous. Existing descriptions are based almost entirely on morphology of adult specimens, especially on position of the genital pore and extent of the vitellaria. Some species are based on the description of a single individual, without adequate consideration for the variation that always and inevitably occurs. It is admitted that morphological divergence results from differences in age and degree of maturity, from extension and retraction of entire specimens or of particular regions of the body, from the accumulation of reproductive products and from procedures of examination, fixation and preservation, especially the degree of flattening under pressure of a coverglass. Measurements made on living specimens may differ significantly from those made on the same individuals after fixation and staining. Moreover, although specificity in the molluscan host may be relatively restricted, representatives may develop in final hosts as diverse as birds and mammals, with substantial structural modifications. *Paramonostomum echinatum* Harrah, 1922 and *Paramonostomum pseudalveatum* Price, 1931, were described from muskrats, *Ondatra zibethica,* whereas all other species are from avian hosts. Swales (1933) reported, without description, the finding of *P. pseudalveatum* in *Branta canadensis* taken in Nova Scotia. Lal (1936) included *P. parvum* in a new genus, *Neoparamonostomum,* based on *Paramonostomum ionorne* Travassos, 1921 and characterized by the location of the genital pore and extent of vitellaria. Harwood (1939) discussed the genus *Paramonostomum,* suppressed *Neoparamonostomum* as a synonym of *Paramonostomum,* and considered the problems of generic and specific identity. He noted that *Paramonostomum* differs from other notocotylid genera only in the absence of ventral glands, and since these glands are frequently very difficult to locate, it is possible that some of the species now assigned to *Paramonostomum* may ultimately be found to belong elsewhere. The species of *Paramonostomum* were arranged in two groups: the *Alveatum* group, short, oval, with vitellaria extending to the level of the cirrus sac, to contain the species, *alveatum, pseudalveatum, parvum,* and possibly *ionorne;* and the *Elongatum* group, with elongate, spatulate bodies, suggestive of *Notocotylus,* with a space between the vitellaria and the cirrus sac, to contain all the other species. Although the observation of Harwood has merit, Dunagan (1957, p. 581) commented, "Neither of these groups, however, possesses characters inherent to one but not both. The division is, therefore, of little value for systematic purposes." Harwood supplemented the description of *P. parvum* by the study of specimens from the Helminthological Collection of the U. S. National Museum, including No. 39598 from the intestine of a blue goose, *Chen caerulescens,* collected by A. M. Fallis in Ontario, Canada, and No. 43148 from the intestine of the American golden-eye, *Glaucionetta clangula americana,* collected by D. K. Coburn at the Migratory Bird Refuge, Brigham, Utah. The worms measured 0.69 to 0.80 mm. in length and 0.46 to 0.50 mm. in width. Harwood stated (p. 337), "The specimens on which the present redescription is based are, if judgment is based on size alone, more similar to *Paramonostomum alveatum* than to *P. parvum.* They are referred to the latter species, because in the writer's opinion size is an extremely variable character in trematodes, and because both the distribution of the vitellaria and position of the genital pore are as described and figured by Stunkard and Dunihue; they differ in these respects from *P. alveatum* as figured by both Lühe (1909) and Kossack (1911). These structural characters are regarded as more important than size differences." Harwood assigned the species reported by Swales (1933) to *P. parvum.*

The studies of Harwood raise questions of specificity in the genus *Paramonostomum.* The position of the genital pore relative to the bifurcation of the digestive tract, and the extent of the vitellaria are variable; indeed, Harwood observed (p. 336), that the location of the genital pore "may not be wholly reliable in specimens preserved in a contracted state, especially if the cephalic end is curved ventrad." Observation of living specimens discloses much variation in location of the genital pore as the anterior end of the body is extended and retracted. The description of *P. alveatum* by Kossack (1911) was based on material from a number of host species and the specimens varied from 0.78 to 0.90 mm. in length and 0.50 to 0.56 mm. in width. The genital pore was described as ventral to the intestinal bifurcation and the vitellaria occupied the middle third of the body. Concerning the vitellaria, Kossack stated (p. 564), "Doch ist ihre Erstreckung nicht ganz konstant, da sie häufig nach hinten bis zum Vorderrand der Hoden reichen." In the present study, the cercariae, metacercariae, and adults are referable to two different size groups. The adults of one group are less than 0.50 mm. in length and are identified as *P. parvum;* adults of the other group are 0.55 to 0.90 mm. in length and are identified as *P. alveatum.* If there were specimens of intermediate size, it would be feasible to include all in a single species, but since all were from the same intermediate host-species and developed in the same final host-species, the differences appear to be genetic. The specimens studied by Harwood agree with the larger of the present species and may belong to *P. alveatum.*

Examination of published descriptions in the light of the above considerations raises doubt concerning the validity of certain species. *Paramonostomum pseudalveatum* Price, 1931, from the muskrat, is very similar to *P. parvum* Stunkard and Dunihue, 1931, from an unidentified duck, whose life-cycle is reported in the present paper. The two species are virtually equal in size and shape; in both the cirrus sac is short and wide, with loops of the seminal vesicle and uterus extending beside the sac; the metraterm is short, not more than one-half the length of the cirrus sac; the vitellaria extend from the testes to the level of the cirrus sac, and the uterus has 8 to 11 transverse loops. *Paramonostomum pseudalveatum* has a larger oral sucker, larger gonads, larger cirrus sac and a somewhat more anterior location of the genital pore. If these features are the result of development in different hosts, the two species may be identical. Similarly, *Paramonostomum brantae* Bullock, 1952, agrees so completely with descriptions of *P. alveatum* that the two specific concepts merge and *P. brantae* falls in synonymy. Bullock (1952) noted the similarity and distinguished between the two species on the shape of the ovary in *P. alveatum,* which he recognized as an unreliable character, and the larger cirrus sac of *P. brantae.* The figure of *P. brantae* shows the cirrus sac expanded and filled with spermatozoa. *Paramonostomum macrostomum* Ku, 1938, was described on a single specimen from *Fulica atra* taken at Soochow, China. A somewhat larger single specimen from the same host, *F. atra,* taken at Lucknow, India, was described by Baugh (1958) as *Paramonostomum fulicai. Paramonostomum nettioni* Baugh, 1958, from the common teal, *Nettion crecca,* is similar morphologically and is intermediate in size between *P. macrostomum* and *P. fulicai,* but information is inadequate to determine the specific status of these species. Two species, *Paramonostomum casarcum* from *Casarca rutila* and *Paramonostomum querquedulum* from *Querquedula circia,* were described by Lal (1936) in India. Each species was described from a single specimen. The worms are approximately the same size and morphological agreement is so complete and precise that specific distinction is highly questionable. An item of reported difference is the location of the genital pore, which in *P. casarcum* is at the posterior border of the oral sucker, whereas in *P. querquedulum* it is slightly anterior to the intestinal bifurcation. But the location of the pore shifts with extension and retraction of the anterior end of the body and with the orientation of the oral sucker. If the sucker is turned so that the mouth is subterminal, the esophagus appears short and bent and the pore apparently is farther forward. Moreover, the two species described by Lal (1936) are very similar to and may be identical with worms from ducks taken at Soochow, China, and described by Hsü (1935) as *Paramonostomum ovatum.* The description of *Paramonostomum microstomum* by Moghe (1932) is incomplete, the uterus is represented in diagrammatic manner and the locations of the ovary and Mehlis' gland are reversed. The specimens were from *Philomachus pugnax,* taken at Nagpur, India, and are similar to those described by Lal (1936). The single specimen from *Querquedula discors* taken in Mexico and described by Caballero (1942) as *Paramonostomum obtortum,* closely resembles the worms described by Lal (1936). A distinct group, characterized by long cirrus sac and short vitelline zones, includes *Paramonostomum actiditis* Cable, 1960, from charadriiform birds of Puerto Rico, and *Paramonostomum histrionici* Ching, 1962, from *Histrionicus pacificus* taken near Friday Harbor, Washington. Three other species are characterized by linear, spatulate bodies, very long cirrus sacs that extend to the middle of the body and short vitelline zones. They are *Paramonostomum elongatum* Yamaguti, 1934, from *Olor bewicki jankowskii* and *Olor cygnus* taken in Korea; *Paramonostomum bucephalae* Yamaguti, 1935, from *Bucephala clangula, Tadorna tadorna, Spatula clypeata* and *Nyroca marila mariloides,* taken in Japan; and *Paramonostomum malerischi* Dunagan, 1957, from the emperor goose, *Philacte canaganica,* taken in Alaska. The description of new species from single specimens is not commended and final determination of specific identity in the genus *Paramonostomum* may depend on the discovery of life-cycles and the description of larval stages.

SUMMARY

The account of Mme. Kulachkova (1954) on the life-history of *Paramonostomum alvealtum* is confirmed. The asexual generations and larval stages of both *Paramonostomum alveatum* and *Paramonostomum parvum* occur in the prosobranchiate snail, *Hydrobia salsa,* found in brackish-water ponds near Woods Hole,

Massachusetts. Sexually mature worms have been obtained by feeding metacercariae to day-old chicks and laboratory-reared eider and domestic ducklings. Adult and larval stages of both species are described and figured. Problems of specific identity in the genus *Paramonostomum* are discussed.

LITERATURE CITED

Baugh, S. C., 1958. Contributions to our knowledge of digenetic trematodes. III. *Proc. Nat. Acad. Sci. India, Sect. B,* **28**: 205–226.

Bullock, W. L., 1952. Two new species of monostomes from the Canada goose with a review of *Paramonostomum alveatum* (Mehlis in Creplin, 1846). *J. Parasitol.,* **38**: 371–378.

Caballero y Caballero, E. 1942. Descripción de un *Paramonostomum* (Trematoda: Notocotylidae) encontrado en los patos silvestres del lago de Texcoco, V (1). *Anal. Inst. Biol.,* **13**: 91–95.

Cable, R. M., 1960. Digenetic trematodes of Puerto Rican shore birds. *Sci. Survey Porto Rico and Virgin Islands; New York Acad. Sci.,* **17** (2): 191–255.

Ching, Hilda L., 1961. Three trematodes from the harlequin duck. *Canad. J. Zool.,* **39**: 373–376.

Dunagan, T. T., 1957. *Paramonostomum malerischi* n. sp. (Trematoda: Digenea: Notocotylidae) from the emperor goose, *Philacte canagica* L. in Alaska. *J. Parasitol.,* **43**: 586–589.

Harrah, E. C., 1922. North American monostomes primarily from freshwater hosts. *Illinois Biol. Mongr.,* **7**: 225–324.

Harwood, P. D., 1939. Notes on Tennessee helminths. IV. North American trematodes of the subfamily Notocotylinae. *J. Tenn. Acad. Sci.,* **14**: 332–341, 421–437.

Hsü, Y. C., 1935. Trematodes of fowls in Soochow. *Peking Nat. Hist. Bull.,* **10**: 141–150.

Kossack, W., 1911. Über Monostomiden. *Zool. Jahrb., Syst.,* **31**: 491–590.

Ku, C. T., 1938. New trematodes from Chinese birds. *Peking Nat. Hist. Bull.,* **13**: 129–136.

Kulachkova, V. G., 1954. The life cycle and pathogenic significance of the trematode, *Paramonostomum alveatum* (Mehlis, 1846). Trudi Problemnikh i Trematisheskikh Soveshchani. Akad. Nauk SSSR. No. 4: 118–122. (In Russian)

Kulachkova, V. G., 1961a. Annual and seasonal variations in infection of mollusks by larvae of *Paramonostomum alveatum* (Trematoda). Trudi Karelskogo Filiala Acad. Nauk SSSR. No. 30: 79–89. (In Russian)

Kulachkova, V. G., 1961b. The biology of the larval stages of *Paramonostomum alveatum* (Trematoda) a parasite of the eider. Trudi Karelskogo Filiala Akad. Nauk SSSR. No. 30: 90–91. (In Russian)

Lal, M. B., 1936. A review of the genus *Paramonostomum* Lühe, with descriptions of two new species and remarks on the genera of the subfamily Notocotylinae. *Proc. Ind. Acad. Sci.,* **3**: 25–34.

Lühe, M., 1909. Parasitische Plattwürmer. I. Trematoden. *In:* Süsswasserfauna Deutschlands. Heft 17: 215 pp.

Moghe, M. A., 1932. Two new species of trematodes from an Indian ruff, *Philomachus pugnax* Gray. *Parasitol.,* **24**: 54–59.

Monticelli, F. S., 1892. Studii sui trematodi endoparassiti; sul genere *Notocotyle* Diesing. *Boll. Soc. d. Nat. Napoli. 1 s.,* **6**: 26–46.

Odening, K., 1966. Physidae und Planorbidae als Wirte in den Lebenszyklen einheimischer Notocotylidae (Trematoda: Paramphistomidea). *Zeitschr. Parasitenk.,* **27**: 210–239.

Pilsbry, H. A., 1905. A new brackish-water snail from New England. *The Nautilus,* **19**: 90–91.

Price, E. W., 1931. Four new species of trematode worms from the muskrat, *Ondatra zibethica,* with a key to the trematode parasites of the muskrat. *Proc. U. S. Nat. Museum,* **79**: 1–13.

Rothschild, Miriam, 1935. Note on the excretory system of *Cercaria ephemera* Lebour, 1907 (*nec* Nitsch). *Parasitol.,* **27**: 171–174.

Rothschild, Miriam, 1938. Notes on the classification of cercariae of the superfamily Notocotyloidea (Trematoda), with special reference to the excretory system. *Novitat. Zool.*, **41**: 75–83.

Rothschild, Miriam, 1941. Note on the life history of the genus *Paramonostomum* Lühe, with special reference to the excretory vesicle. *J. Parasitol.*, **27**: 363–365.

Stunkard, H. W., 1960. Studies on the morphology and life-history of *Notocotylus minutus* n. sp., a digenetic trematode from ducks. *J. Parasitol.*, **46**: 803–809.

Stunkard, H. W., 1965. Studies on trematodes of the family Notocotylidae. *Biol. Bull.*, **129**: 425.

Stunkard, H. W., 1966a. Further studies on digenetic trematodes of the family Notocotylidae. *Biol. Bull.*, **131**: 409.

Stunkard, H. W., 1966b. The morphology and life-history of *Notocotylus atlanticus* n. sp., a digenetic trematode of eider ducks, *Somateria mollissima*, and the designation *Notocotylus duboisi nom. nov.* for *Notocotylus imbricatus* (Looss, 1893) Szidat, 1935. *Biol. Bull.*, **131**: 501–515.

Stunkard, H. W., and F. W. Dunihue, 1931. Notes on trematodes from a Long Island duck with description of a new species. *Biol. Bull.*, **60**: 179–186.

Swales, W. E., 1933. A review of Canadian helminthology. *Canad. J. Res.*, **8**: 468–482.

Szidat, L., and U. Szidat, 1933. Beiträge zur Kenntnis der Trematoden der Monostomidengattung *Notocotylus* Dies. *Zentralbl. Bakt. Parasitenk., Abt. 1,* **129**: 411–422.

Yamaguti, S., 1934. Studies on the helminth fauna of Japan. Part 3. Avian Trematodes II. *Japan J. Zool.*, **5**: 543–583.

Yamaguti, S., 1935. Studies on the helminth fauna of Japan. Part 5. Trematodes of birds III. *Japan J. Zool.*, **6**: 159–182.

Yamaguti, S., 1938. Zur Entwicklungsgeschichte von *Notocotylus attenuatus* (Rud., 1809) und *N. magniovatus* Yamaguti, 1934. *Zeitschr. Parasitenk.*, **10**: 288–292.

Vol. 132, No. 2 April, 1967

THE

BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

NORTHERN PACIFIC GIGANTIONE (ISOPODA)

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While carrying out research on the crabs of Eniwetok Atoll, Dr. Jens Knudsen of Pacific Lutheran University noticed a laterally distorted specimen in the collection of the Eniwetok Marine Biological Laboratory. He very kindly sent this crab to the writer. The animal had been identified by Dr. J. Garth of the University of Southern California, and had been collected by Dr. A. H. Banner of the University of Hawaii. The host (Fig. 1A) had an isopod located in the left gill area, and this ectoparasite was found to be a member of the Bopyridae family in the Epicaridea suborder.

Some years ago the first record of a shore bopyrid for the Hawaiian Islands was reported by Danforth (1963). At that time an exact identification was not made; however, the assumption was that the form was in the "Ione" or "Cepon" group. Verification of the former hypothesis may be aided by comparison with the first bopyrid to be reported from Eniwetok Atoll. This new form is in the genus *Gigantione,* and it and the Hawaiian specimen related to two species of *Gigantione* reported by Shiino (1941, 1958). Thus it now appears that there are four species of the genus in the northern Pacific, two of which have been previously undescribed.

Genus *Gigantione* Kossmann 1881

Gigantione pratti n. sp.

Material: one pair.
Host: *Phymodius ungulatus* (Milne Edwards). Parasitized in the left branchial region.
Locality: Bruce (Aniyaanii) Island, Eniwetok Atoll. Approximately 162° 28′ E. Long., 11° 28′ N. Lat., in dead *Acropora* coral, at a depth of about 6 feet.
Date: collected on 23 February 1957.

FEMALE

Dimensions: 5.0 mm. greatest length, excluding lamellae; 4.5 mm. greatest width, at the third thoracomere.

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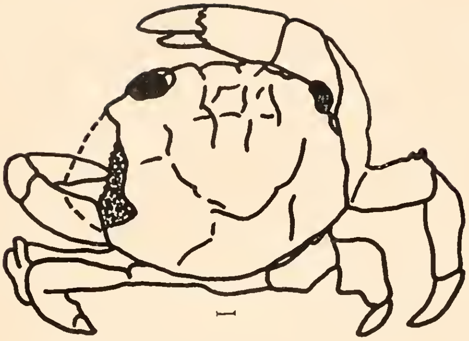

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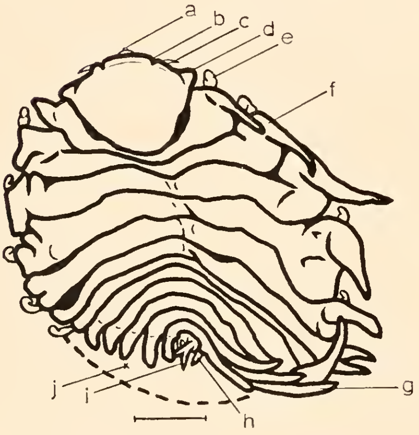

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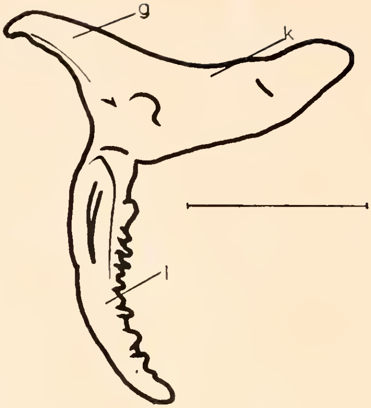

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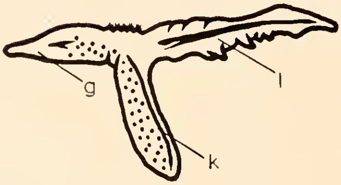

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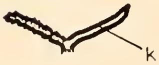

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All sketches, except that of the crab, are of *Gigantione pratti* n. sp. Unless otherwise indicated, each sketch was drawn by means of a camera lucida. Where a bar (I) is shown, it represents 1 millimeter.

Figure 1a. *Phymodius ungulatus,* drawn from a photograph. The portion of the carapace which was removed is indicated by a dotted line.

Figure 1b. Dorsal aspect of the female bopyrid. The extent of the marsupium is indicated by a dotted line.

Figure 1c. First right pleopod of the female, with adjacent epimere. Drawn from a photomicrograph.

Figure 1d. First left pleopod of the female, with adjacent epimere.

Figure 1e. Pleopod #4 of the female.

Cephalon. Comprising 1 large lobe, deeply sunken into the thorax, with 2 ear-like processes. There is a barely discernible anterior border or velum on the head, having a notch just antero-medial to each of the processes. No eyes. No pigmentation. The tip of the oral cone can just be seen from the dorsal aspect of the parasite.

Thorax. Seven segments, with no pigment, and with only moderate axial flexion. Most of the distortion to the right is due to unequal growth of the two sides (Fig. 1B). All the pereopods are present. The oöstegites do not completely cover the marsupium, leaving a slight gap at the third and fourth thoracomeres. There is no hook on the first incubatory lamella (Fig. 2F), although a slight ridge is present. Obvious, finger-like coxal plates are on the right side, being larger from segments 1 through 3, and then decreasing through segment 7. The coxal plates of the left side range posteriad from blade-like to anvil-shaped.

Abdomen. Considerably foreshortened, being about one-fifth of the total body length, and hidden to a large extent ventrally by the swollen marsupium. Six segments evident, the sixth having 2 "Y-shaped" uropods (Fig. 2G). There are 5 pairs of pleopods, each pair being biramous, and having the exopods of numbers 2 through 5 relatively smooth and elongate, while the corresponding endopods are long, thin, and slightly tuberculated (Fig. 1E). The first pair of pleopods on either side (Figs. 1C, 1D) are much larger than the others, and tend to cover them. The exopodite is smooth and blade-like, whereas the endopodites are tuberculated or somewhat pinnately divided at the border. Each biramous pleopod is adjacent to a smooth-edged, elongated epimeral plate.

MALE

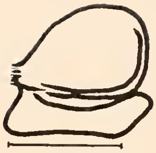
Dimensions: Length 2.5 mm. Width 1.0 mm. at the fourth thoracomere.

Location. On the smaller side of the ventral abdomen of the female, with the male's head in the same direction as that of its mate. Although the male was not within the marsupium (Fig. 1B), it was covered by the bulbous oöstegites.

Cephalon. Blunt, with neither a border nor any processes. Eyes distinct, with a reddish cast. First antenna of 2 articles, second antenna of 4 articles, tipped with bristles. No pigment.

Thorax. Typical fusiform shape, widest at the fourth segment. Lateral plates not unusual in appearance. Seven distinct segments and 7 pairs of pereopods. No pigment.

Abdomen. Five separate, tapering segments, plus the telson. No pigmentation. The lateral plates gently rounded, except the fifth pair, which is hooked posteriad. Five pairs of pleopods, each with a short and a longer ramus (Fig. 2I). The rami are tube- or rod-like, and lie almost transversely to the abdominal axis. The short ramus of each is lateral, with the longer ramus nearly meeting its counterpart at the abdominal midline. The uropods are 2 in number, heart-shaped, with the pointed end anteriorly. There is a posterior indentation on each which is quite evident (Fig. 2J), rather than being merely a slight notching. The uropods are plainly visible from the dorsal aspect of the male (Fig. 2H), and are slightly roughened, but not hirsute.



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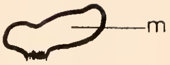

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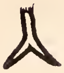

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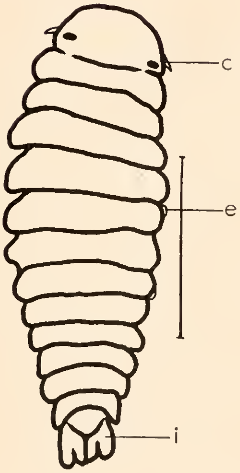

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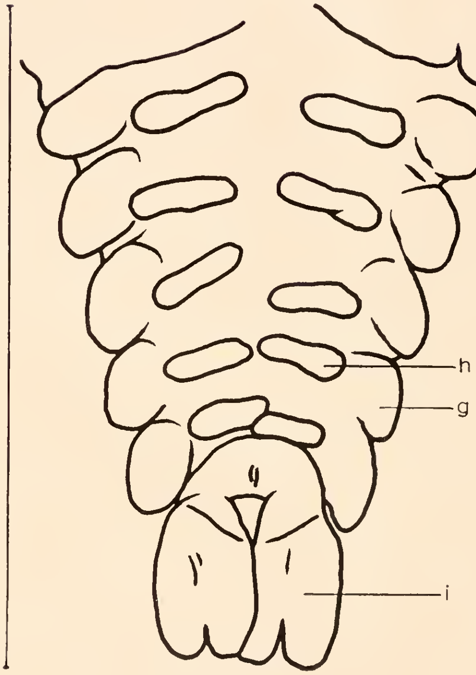

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Figure 2F. Internal face of the first oöstegite of the female.
Figure 2G. Right uropod of the female.
Figure 2H. Dorsal aspect of the male bopyrid. Drawn from a photomicrograph.
Figure 2I. Second right pleopod of the male.
Figure 2J. Ventral aspect of the abdomen of the male. Drawn from a photomicrograph.

a = oral cone, b = notch in marginal velum, c = antenna, d = cephalic process, e = pereopod, f = coxal plate, g = abdominal lamella, h = pleopod, i = uropod, j = position of male, k = exopodite, l = endopodite, m = medial ramus.

REMARKS

As can be seen from the accompanying tables, *G. pratti* differs from all males in the genus by the form of the uropoda. The male pleopods are similar to those of *sagamiensis,* but there is pigmentation and a pair of uniramous, foliaceous uropods

TABLE I

Major characteristics of Gigantione species

Name, size, locale, host	Head	Thorax and appendages	Abdomen and appendages	Uropoda
bouvieri ♀ = 3–4 mm. ♂ = 0.5? mm. Azores, in *Pilumnus* W. Indies, in *Hypoconcha*	"Ear-like" processes. ♂ No eyes.	Coxal plates narrow and folded. Plates on all 7 segments	1st 2 pleomeres fused in midline. Pleomeres 1–5 with straight prolongations. Pleopods almost triramous, 2–5 tuberculated. 1st pair largest. ♂ Pleopods 1–5 rod-like.	Shaped like a two-fingered glove. ♂ Pea-shaped.
giardi ♀ = 20 mm. ♂ = 7 mm. Tuamoto, in *Xantho*	Processes. Large, with fleshy border. ♂ Eyes.	2 "saillies" on anterior portions of each segment. Lateral plates on 1–7. 5 pairs large oöstegites, #1 with internal ridge.	Plates 1–5 like those of the pereon. #6 with 2 lamellae plus uropods. Pleopods like those of *moebii*. ♂ Pleopods = "laminaires."	Fleshy base with 2 small cylindrical branches. ♂ 2 leaf-like plates.
hawaiiensis ♀ = 9.7 mm. ♂ = 3.4 mm. Hawaii, in *Xantho*	Bilobed at posterior. Processes. ♂ Eyes. Slight pigmentation.	Long, thick lamellae or coxal plates on one side, triangular on other side. ♂ Lateral pigmentation.	Long, thin lamellae on 1–5. Pleopods subtriramous; more pinnate than tuberculated. ♂ Pleopods uniramous rods. Slight pigmentation.	Narrow base with 2 elongate, finger-like branches. ♂ Kidney-shaped and pubescent.
ishigakiensis ♀ = 13 mm. ♂ = 3.2 mm. Japan, in *Carpilius*	Processes. Frontal lamina. ♂ Eyes. No pigment.	Elongate, tapering coxal plates. Closed marsupium. ♂ Widest at segment 6.	Lamellae on 1–5 like coxal plates. Pleopod 1 largest, lamellar; digitiform processes on both rami. 2–5 are heavily tuberculated. ♂ 5 pairs of rod-shaped, uniramous pleopods.	Shaped like a 2-fingered glove. ♂ Uniramous, foliaceous.
moebii ♀ = 15 mm. ♂ = 3? mm. Isle Maurice, in *Ruppelia*	No margin. Processes. ♂ Eyes. No pigment.	Marsupium covered. Coxal plates heavy, but not long.	Small pleon plates. Pleopod 1 triramous, relatively smooth. Pleopods 2–5 triramous, with heavy tuberculation. ♂ Pleopods 1–5 egg-shaped.	Swollen base with 2 thin branches. ♂ Uniramous and leaf-like.

TABLE I (continued)

Name, size, locale, host	Head	Thorax and appendages	Abdomen and appendages	Uropoda
pratti ♀ = 5.0 mm. ♂ = 2.5 mm. Eniwetok, in *Phymodius*	Processes. Notched anterior margin. ♂ Eyes. No pigment.	Marsupium slightly open. Coxal plates thin, long on larger side; triangular on smaller side.	Pleomeres 1–5 with blade-like plates. Pleopod 1 largest, biramous. Endopodites of other pleopods slightly tuberculated. ♂ 5 pairs of biramous, rod-like pleopods.	"Y-shaped," with a narrow base. ♂ Cordate, but still uniramous.
rathbunae ♀ = 4 mm. ♂ = 1 mm. "Salomon Islands," in *Actaea*	No margin. No processes. Eyes. ♂ Eyes. No pigment.	Coxal plates thin and finger-like. Segments 1–4 double on the left.	Plates large and finger-like on large side; triangular on small side. Pleopod 1 largest, with marginal serrations. ♂ 5 pairs of bulbous pleopods.	Swollen base with 2 tapered rami. ♂ Uniramous, foliaceous and hirsute.
sagamiensis ♀ = 3.7 mm. ♂ = 1.4 mm. Japan, in *Carpiliodes*	No margin. No processes. ♂ Eyes.	Coxal plates from slightly pointed to crescentic and blunt. Marsupium almost closed.	Plates 1–5 tuberculated and folded back. 5 pairs of biramous pleopods: endopod is filiform, tuberculated; exopod more blunt. Pleopod 1 is largest, nearly triramous. ♂ Pigmented. 5 pairs of rod-shaped, biramous pleopods.	Swollen base with 2 short, blunt rami. ♂ Uniramous, leaf-like.

on the latter. The *G. pratti* female has ear-like cephalic processes, as do all other species except *rathbunae* and *sagamiensis;* however in these, neither has a cephalic margin, and in the case of *rathbunae,* eyes are present. The female *bouvieri,* as illustrated by Nierstrasz and Brender à Brandis (1931), has structurally different coxal plates, and a partial fusion of pleomeres 1 and 2. The female *giardi* has a fleshy cephalic border, and lamellae on pleomere 6. The female *ishigakiensis* has a different frontal lamella, a closed marsupium, and digitiform processes on the exopodite of the first pleopod. The female *moebii* lacks a cephalic margin, has differently shaped coxal plates, shorter abdominal lamellae, and a triramous condition for pleopod 1. Aside from the foregoing differences, the female *pratti* seems to be unique in the possession of "Y-shaped" uropods. The uropoda of other females of the genus are biramous, but the branches range from short and separated to fairly long and distinct; in none do they diverge abruptly from a narrow base.

The male allotype, and female holotype have been deposited in the United States National Museum, catalog number 113940. The host crab is catalog number 113939.

Named for Dr. Ivan Pratt, parasitologist at Oregon State University, who first suggested to the writer that the epicarid isopods might be an interesting field of study.

Gigantione hawaiiensis n. sp.

For added data and sketches, refer to Danforth (1963).
Material: one pair.
Host: *Xantho crassimanus* Milne Edwards. Parasitized in the left branchial region.
Locality: tide pool at Diamond Head, Oahu, Hawaii.
Date: collected on 13 January 1962.

FEMALE

As described. The female is stated to have 6 pairs of abdominal lamellae, while the drawing shows but 5 pairs.

MALE

As described. The pleopods are referred to as tubercles.

REMARKS

A reconsideration of this previously described Hawaiian form seems to indicate that the genus is correctly *Gigantione*. The structure of the male, and some of the features of the female, make it obvious that it does not belong in one of the existing species. The "claw-shaped" appendages which were found free in the preservative undoubtedly are the uropoda of the female.

The accompanying tabulation indicates the differences between *G. hawaiiensis* and other species of the genus. The partially bilobed head of the female is unique, and the pinnate structure of the pleopod rami is in contrast to the more commonly found tuberculations of other forms. The male has pigmentation, as opposed to others except *sagamiensis,* and the disc-like uropoda are distinctly at variance with the foliaceous, pea-shaped, or cordate shapes illustrated by other males.

The specimens have been deposited in the United States National Museum, catalog numbers: 110192 (larvae), 110191 (female holotype), and 110190 (male allotype).

Discussion

As stated by Bonnier (1900, p. 276) for *Gigantione*: "Deux caractères suffisent à caractèriser ce genre: la femelle adulte possède des lames pleurales sur tous les somites, tant ceux du thorax que ceux de l'abdomen, et ses uropodes sont biramés." The species described so far are:

- *G. bouvieri* Bonnier
- *G. giardi* Nobili
- *G. hawaiiensis* n. sp
- *G. ishigakiensis* Shiino (1941)
- *G. moebii* Kossmann
- *G. pratti* n. sp.
- *G. rathbunae* Stebbing
- *G. sagamiensis* Shiino (1958)

The major characteristics of these species are shown on the accompanying tabulation (all female forms have 7 thoracomeres, 6 pleomeres, a single-lobed head, and biramous uropoda). Reference was made to *Paragigantione papillosa* Barnard, since it is only a matter of degree between: "Eine Anzahl Coxalplatten am Pereion sehr entwickelt (odor sehr abweichend gestaltet)," and, "Alle Coxalplatten am Pereion nur mässig entwickelt bis fehlend," as used in the key by Nierstrasz and Brender à Brandis (1932, pp. 90, 91). However, the two genera are quite dissimilar in many respects, so *Paragigantione* is not included in the table.

In reviewing almost any of the information on epicarids, one finds many instances of contradiction or confusion. Some points are purely typographical errors, others misinterpretation, etc. A disconcerting item for *G. giardi* is Nobili's (1906, p. 270) statement: "Lames pleurales des segments de l'abdomen conformées comme celles du thorax; sixième segment pourvu aussi de deux lamelles, et de petits uropodes charnus." If correct, this is entirely different from all other species in the genus, and might lead one to place the form into the genus *Orbione*. Unfortunately, there is no illustration against which the description could be checked (such as is the case in Bonnier's description of *G. moebii* in which he mentions the presence of 6 pairs of pleopods, while his drawing shows 5 pairs). Further ambiguity is encountered in a key by Dakin (1931), wherein *Crassione* is separated from *Gigantione* on the basis of the uropods of the former being biramous, whereas those of the latter are given as uniramous! Since *Gigantione* has biramous uropoda, it is indeed fortunate that sketches of *Crassione* indicate that the specimen is in fact not *Gigantione*. It is points such as these, coupled with accidental mislabeling or identification, that indicate the great need for an evaluation of the available literature on epicarids.

SUMMARY

1. Eight species of *Gigantione* have now been described. One was from the north Atlantic, one from the Indian Ocean, two from the south Pacific, and four from the north Pacific. Of these last, *G. pratti* and *G. hawaiiensis* are new species. The hosts of the different species have all been in separate genera, with the exception of those for *G. giardi* and *G. hawaiiensis,* both of which were in *Xantho*.

2. Dr. Shiino is carrying out an intensive investigation of epicarids in the Japanese archipelago, and the writer is in the process of preparing a monograph covering the Epicaridea of the northern Pacific (except for those areas and forms near Japan). Therefore, it should be expected that many new species and possibly genera will be found in the Pacific as collecting continues.

LITERATURE CITED

BARNARD, K. H., 1920. Contributions to the crustacean fauna of South Africa. Number 6. Further additions to the list of marine Isopoda. *Ann. So. African Mus.*, **17**: 319–438.

BONNIER, J., 1900. Contribution à l'Étude des Épicarides: Les Bopyridae. *Trav. Stat. Zool. de Wimereux*, **8**: 1–475.

DAKIN, W. J., 1931. On a new bopyrid parasite from the coast of New South Wales. *Proc. Linn. Soc. New South Wales,* **56**: 267–272.

DANFORTH, C. G., 1963. First record of a Hawaiian shore bopyrid (Isopoda: Bopyridae). *J. Parasit.,* **49**: 847–850.

KOSSMANN, R., 1881. Studien über Bopyriden. I. *Gigantione moebii* und Allegemeines über die Mundwerkzeuge der Bopyriden. *Zeitschr. f. wiss. Zool.,* **35** : 652–665.

NIERSTRASZ, H. F., AND G. A. BRENDER À BRANDIS, 1931. Papers from Dr. Th. Mortensen's Pacific Expedition 1914–16. Epicaridea II. *Viden. Medd. fra Dansk naturhist. Foren. København,* **91** : 147–226.

NIERSTRASZ, H. F., AND G. A. BRENDER À BRANDIS, 1932. Alte und neue Epicaridea. *Zool. Anz.,* **101** : 90–100.

NOBILI, G., 1906. Diagnoses préliminaires de Crustacés, Décapodes et Isopodes nouveaux recueilles par M. le Dr. G. Suerate aux Iles Touamotou. *Bull. Mus. Hist. Nat., Paris,* **12**: 256–270.

SHIINO, S. M., 1941. Further notes on bopyrids from Kyûsyû and Ryûkyû. *Annot. Zool. Jap.,* **20**: 154–158.

SHIINO, S. M., 1958. Note on the bopyrid fauna of Japan. *Rept. Fac. Fish. Pref. Univ. Mie,* **3**: 29–73.

STEBBING, T. R. R., 1910. Isopoda from the Indian Ocean and British East Africa. *In:* Reports of the Percy Sladen Trust Expedition to the Indian Ocean in 1905. *Trans. Linn. Soc. London, ser. 2, Zool.,* **14**: 83–118.

SURFACE AREA RESPIRATION DURING THE HATCHING OF ENCYSTED EMBRYOS OF THE BRINE SHRIMP, ARTEMIA SALINA

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When the encysted embryos of *Artemia salina* are placed in water (hydration) the embryos resume development. After an interval of time depending upon conditions of incubation, excystment takes place in two stages. The first stage (emergence) occurs when the hard outer cyst wall splits, and the embryo emerges headfirst within a hatching membrane. The second stage (hatching) occurs a few hours later when a nauplius larva swims from the membrane and shell. The transition from the encysted stage to the emerged stage depends upon an uptake of water, mainly due to increased internal concentration of glycerol (Clegg, 1964) and possibly to an increase of free amino acids at the same time (Emerson, 1967). The uptake of water increases the volume of the developing embryo to cause the cyst shell to split. There is consequently an increase of the surface area of the embryo which is shown by scaled micro-photographs of Nakanishi *et al.* (1962).

There are several studies of respiration of *Artemia* during development (Urbani, 1946; Dutrieu, 1960; Muramatsu, 1960; Emerson, 1963; Clegg, 1964). These studies are difficult to compare because of different sources of cysts, possible differences in percentage of viable cysts, and experimental differences in the salinity and temperature of the hatching solution. In spite of differences reported for the rate of oxygen consumption of the embryos, some of these studies reveal a similar pattern. The oxygen consumption rate increases rapidly within the first few hours after hydration, and then remains constant for a time. A second increase occurs at about the time of emergence. Von Bertalanffy and Krywienczyk (1953) have shown that oxygen consumption of the nauplius and later stages of *Artemia* is proportional to surface area. An increase of surface area during emergence could account for the increase of respiration which occurs at the same time. The present study demonstrates that oxygen consumption patterns of developing *Artemia* embryos can be interpreted on the basis of surface rule respiration.

MATERIALS AND METHODS

1. *Source of encysted Artemia embryos*

The encysted embryos used in this study were obtained in 1965 from Ward's of California (Monterey). The cysts were from Great Salt Lake, Utah.

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[1] Supported in part by NIH predoctoral fellowship (1-F1; Gm-21.084) while the author was at the University of South Dakota, Vermillion.

2. *Respiration measurements*

Oxygen consumption of the *Artemia* embryos was measured with a Warburg constant volume respirometer (Umbreit *et al.,* 1959). Dry cyst samples weighing 10.0 mg. were placed in flasks (18 ml. volume) with center well and sidearm. The flasks contained 2.5 ml. 0.5 *M* NaCl, and 0.2 ml. 20% KOH in the center well. Readings were made at 1–2-hour intervals at 25° C. Calculations are expressed as μl. O_2/hr./mg. dry cyst weight. Most of the experiments were carried out with no agitation of the flasks and no antibiotics in the water. Since somewhat different readings were obtained than in a similar set of experiments (Emerson, 1963), other series of measurements were made with agitation at a rate of 60 complete oscillations/min., and with antibiotics in the water (penicillin, 1000 units/ml. and streptomycin, 100 μg./ml., Clegg, 1964). Microbial activity was evaluated at the end of runs in which there were no antibiotics in the water by filtering off the brine shrimp (Whatman #1 paper) and measuring oxygen consumption of the water over a period of several hours.

3. *Measurement of surface area of cysts and of emerged embryos*

All measurements were made with a dissecting microscope fitted with a calibrated eyepiece micrometer. The encysted embryos are spherical in shape. Surface area was calculated directly from measurements of diameter, using the formula for the surface area of a sphere (area $= 12.57\ r^2$, where r = radius). The emerged embroys have a symmetrical shape resembling a pear. Measurements of emerged embryos were drawn on graph paper. Each drawing was divided into sections. Surface areas of the middle sections were calculated using the formula for the curved surface of a right cylinder (area $= 2\pi rh$, where h = altitude). The surface areas of the two end sections were calculated as the curved surface of a right cone (area $= \pi r \sqrt{r^2 + h^2}$). Areas of individual sections were totaled to give the surface area of the emerged embryo.

4. *Percentage of emergence*

The period of time when 50% of the embryos were fully emerged ($T_{50\%}E$) was estimated by periodic counts of the percentage of emerged embryos during development.

RESULTS

Oxygen consumption rates during development are summarized in Table I. The presence of antibiotics in the incubation media, or agitation of the Warburg vessels does not significantly affect oxygen measurements (Table II). The surface areas of encysted and emerged embryos are compared in Table III.

DISCUSSION

The following terms are used to describe the oxygen uptake pattern of *Artemia* embryos during development (Table I). The hydration period is the first rapid uptake of oxygen; the differentiation period is a plateau during which the rate of oxygen consumption remains about the same; the emergence period occurs during the second rise of oxygen consumption rate when most of the embryos are emerging;

TABLE I

Oxygen consumption of developing Artemia embryos in 0.5 M NaCl at 25° C. The values given are µl. O_2/hr/.mg. dry cyst weight. The numbers preceded by ± signs give confidence limits at the 95% level

Hours of development	Oxygen consumption (15 determinations)	* Period of development
0	—	
1–2	0.46 ± 0.08	Hydration
2–4	0.93 ± 0.02	
4–6	1.06 ± 0.05	
6–8	1.05 ± 0.11	
8–10	1.17 ± 0.17	
10–12	1.10 ± 0.17	Differentiation
12–14	1.20 ± 0.03	
14–16	1.01 ± 0.15	
16–18	1.21 ± 0.23	
18–20	1.44 ± 0.23	
20–22	1.66 ± 0.36	** Emergence
22–24	1.73 ± 0.36	
28–30	1.94 ± 0.42	
30–32	2.00 ± 0.49	Hatching
36–38	2.00 ± 0.26	

* See text for explanation.

** The first emerged embryos were seen at 16 hours; $T_{50\%}E$ was at 24 hours.

and the hatching period is when oxygen consumption levels off again after $T_{50\%}E$ Average rates of oxygen consumption of the differentiation and of the hatching period of this study are compared with other studies (Table IV).

Muramatsu's measurements went only to 12 hours of development so that the hatching period probably was not reached. Urbani's measurements probably represent oxygen consumption well past $T_{50\%}E$, since the figure listed under hatching period (Table IV) is a value for 50 hours of developing. These two studies will not be considered in the following discussion.

TABLE II

Comparison of conditions for oxygen consumption measurements of developing Artemia embryos through 20 hr. development in 0.5 M NaCl at 25° C. The numbers in parentheses indicate the number of determinations. The numbers preceded by ± signs give confidence limits at the 95% level

Condition	Total µl. O_2/mg. dry cyst (20 hr.)
*No antibiotics; not agitated (15)	20.8 ± 1.3
Penicillin and streptomycin; not agitated (6)	19.9 ± 3.0
Penicillin and streptomycin; agitated at 60 complete oscillations per minute (6)	19.7 ± 2.2

* Measurement of filtered water at the end of the runs showed very little oxygen consumption due to microbial activity.

TABLE III

Surface area of Artemia embryos during development

Stage	* Surface area (μ^2)
Encysted embryo (differentiation period)	119,415
Fully emerged embryo (hatching period)	205,178
% increase of surface area	172%

* Averages of 10 measurements. Statistical variation is not shown because individual measurements were almost identical.

The increase in oxygen consumption (Table IV) is very similar to the increase of surface area (Table III) during emergence. This observation suggests that the increase of oxygen consumption rate is proportional to an increase of surface area. The pattern of oxygen consumption (Table I) can be interpreted as follows: Oxygen consumption rises during the hydration period (1–3 hours in duration; Iwasaki, 1964) due to reactivation of metabolism of the dormant embryo. The initial rate rises to a constant value which is limited by the surface area of the cyst throughout the differentiation period. During this period, there is no cell division (Nakanishi *et al.*, 1962; Emerson, 1963), no increase of DNA (Bellini, 1960; Emerson, 1963); and no incorporation of tritiated thymidine (Emerson, 1963). Tritiated thymidine is incorporated only after hatching (Emerson, 1964) as cells start to divide (Nakanishi *et al.*, 1962, 1963). The respiratory quotient remains close to 1 during this period, indicating metabolism of carbohydrate (Dutrieu, 1960; Muramatsu, 1960; Emerson, 1963; Clegg, 1964) which is probably trehalose (Dutrieu, 1960). The rate of oxygen consumption increases during emergence, and rises rapidly to a new peak limited by the surface area of the emerged embryo and early nauplius. The respiratory substrate during and after emergence is probably lipid as indicated by lowered respiratory quotients (Dutrieu, 1960; Emerson, 1963), and increase in lipase activity (Bellini and Lavizzari, 1958) and a decrease in total lipids (Dutrieu, 1960; Urbani, 1959).

The present study shows that surface rule respiration can explain the pattern of oxygen consumption during development of encysted *Artemia* embryos. Similar patterns of respiration exist during the embryonic development of a variety of animals (Boell, 1955). It would be interesting to see if surface rule respiration

TABLE IV

Average rates of oxygen consumption of the differentiation and of the hatching period of Artemia

Reference	µl. O_2 consumed per hour		% increase
	Diff. period	Hatch. period	
Table I	1.11 µl./mg.	1.98 µl./mg.	178
Emerson, 1963	1.66 µl./mg.	2.95 µ./mg.	178
Dutrieu, 1960	1.30 µl./mg.	2.23 µl./mg.	172
Muramatsu, 1960	1.03 µl./mg.	1.45 µl./mg.	141
Urbani, 1946	0.00009 µl./cyst	0.00019 µl./cyst	211

applies for these animals, especially for sea urchins which have strikingly similar patterns (Lindahl, 1939; Wright, 1963).

SUMMARY

Oxygen consumption of *Artemia salina* was measured during development in 0.5 *M* NaCl at 25° C. A pattern is seen in which the rate of oxygen consumption increases rapidly within the first few hours after hydration, remains constant for a time, and then increases rapidly again while most of the embryos are emerging. This pattern is dependent upon surface area of the developing embryo. During emergence, the surface area of the embryo increases 172% over the surface area of the encysted embryo. During the same development period, oxygen uptake increases by almost the same factor.

LITERATURE CITED

Bellini, L., 1960. Osservazioni sugli acidi nucleici nello sviluppo di *Artemia salina* Leach. *Ricerca Sci.*, **30**: 816–833.

Bellini, L., and G. S. Lavizzari, 1958. Studio delle lipase nello sviluppo di *Artemia salina* Leach. *R. C. Accad. Lincei*, **24**: 92–95.

Bertalanffy, L. von, and J. Krywienczyk, 1953. Surface rule in crustaceans. *Amer. Nat.*, **87**: 107–110.

Boell, E. J., 1955. Energy exchange and enzyme development during embryogenesis. *In*: Analysis of Development. Edited by Willier, Weiss, and Hamburger. W. B. Saunders, Philadelphia.

Clegg, J. S., 1964. The control of emergence and metabolism by external osmotic pressure and the role of free glycerol in developing cysts of *Artemia salina*. *J. Exp. Biol.*, **41**: 879–892.

Dutrieu, J., 1960. Observations biochimiques et physiologiques sur le développement d'*Artemia salina* Leach. *Arch. Zool. Exp. Gen.*, **99**: 1–133.

Emerson, D. N., 1963. The metabolism of hatching embryos of the brine shrimp, *Artemia salina*. *Proc. S. D. Acad. Sci.*, **42**: 131–135.

Emerson, D. N., 1964. Incorporation of tritiated thymidine by *Artemia salina*. *Proc. S. D. Sci.*, **43**: 90–95.

Emerson, D. N., 1967. Some aspects of free amino acid metabolism in developing encysted embryos of *Artemia salina*, the brine shrimp. *Comp. Biochem. Physiol.*, **20**: 245–261.

Iwasaki, T., 1964. Sensitivity of *Artemia* eggs to the τ-irradiation III. The sensitivity and duration of hydration. *J. Rad. Res.*, **5**: 91–96.

Lindahl, P. E., 1939. Zur Kenntnis der Entwicklungsphysiologie des Seeigeleies. *Zeitschr. vergl. Physiol.*, **27**: 233–250.

Muramatsu, S., 1960. Studies on the physiology of *Artemia* embryos. I. Respiration and its main substrate during the early development of the encysted embryo. *Embryologia*, **5**: 95–106.

Nakanishi, Y. Y., T. Iwasaki, T. Okigaki and H. Kato, 1962. Cytological studies of *Artemia salina*. I. Embryonic development without cell multiplication after the blastula stage in encysted dry eggs. *Annot. Zool. Japon.*, **35**: 223–228.

Nakanishi, Y. Y., T. Okigaki, H. Kato and T. Iwasaki, 1963. Cytological studies of *Artemia salina*. II. Deoxyribonucleic acid (DNA) content and the chromosomes in encysted dry eggs and nauplii. *Proc. Japan Acad.*, **39**: 306–309.

Umbreit, W. W., R. W. Burris and J. F. Stauffer, 1959. Manometric Techniques, 3rd edition. Burgess Publishing Co., Minneapolis, Minn.

Urbani, E., 1946. L'assuzione di 0_2 durante la vita latente ed il passaggio alla vita attiva. *Boll. Soc. Ital. Biol. Sper.*, **22**: 453–456.

Urbani, E., 1959. Protidi glucidi e lipidi nello svilluppo di *Artemia salina* Leach. *Acta Embryol. Morph. Exp.*, **2**: 171–194.

Wright, B. E., 1963. The biochemistry of morphogenesis. *In*: Comparative Biochemistry, volume VI. Edited by Florkin, M. and H. S. Mason. Academic Press, New York.

UTILIZATION OF DISSOLVED EXOGENOUS NUTRIENTS BY THE STARFISHES, ASTERIAS FORBESI AND HENRICIA SANGUINOLENTA

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In the past few years it has become increasingly evident that nutrition in many types of invertebrate animals involves not only the ingestion of solid foods or particulate matter, but also the utilization of dissolved organic materials commonly found in the environment. While speculation on the significance of this latter source of nutrients dates back at least to the work of Pütter (1909), it remained for Stephens and Schinske (1961) to provide the first clear evidence that dissolved materials can be taken up by a wide variety of invertebrates. These workers demonstrated that representatives of 10 phyla (including Echinodermata) could remove glycine from dilute solutions in sea water. Stephens has continued his investigations and further described the uptake of dissolved amino acids and sugars by several forms, notably the coral *Fungia* (Stephens, 1962), various annelids (Stephens, 1963, 1964), and brittle stars (Stephens and Virkar, 1965, 1966).

In the course of my own studies (Ferguson, 1963a, 1963b), I have observed, by the use of autoradiographic methods, that dissolved C^{14}-labeled nutrients (glucose and amino acids) appear to be readily taken up into at least the epidermal tissues of *Asterias forbesi.* I have suggested that this may represent the most important source of nutrients to some of the more isolated superficial tissues of starfishes, and that in species such as *A. forbesi* the epidermal absorptive process may be facilitated "by enrichment of the medium with stray products released from the externally digested food and by scavenging activities of pedicellariae" (Ferguson, 1963a, p. 79).

Most recently, Pequignat (1966) has reported detailed investigations on a number of echinoderms, including *Asterias rubens,* demonstrating digestion of various types of nutritional products on the skin by glandular secretions and migrating coelomocytes. While his observations are basically subjective in nature, he concludes that at least some of the materials which are digested externally are absorbed directly into the epidermis.

At this time, then, it appears that dissolved organic materials are utilized by starfish (and many other invertebrates), and that at least some of the nutrients are taken up directly by the body surface, thus by-passing the digestive tract. Furthermore, it is probable that in various species of echinoderms mechanisms, such as the pedicellariae, have evolved which serve to enhance the availability of dissolved nutrients to the integuments. There are, however, at least several important questions which are as yet unanswered. First, are dissolved nutrients taken up by the digestive tract as well as the epidermis? Second, do epidermally absorbed nutrients

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[1] Supported by NSF grants GB-2209 and GB-4994.

become distributed throughout the body, or can they benefit only the superficial tissues into which they are initially taken up? And third, are there marked differences in the handling of exogenous nutrients by various species of starfishes? The present investigation has been directed toward these three points.

Materials and Methods

The starfish used in these experiments were freshly collected specimens of *Asterias forbesi* and *Henricia sanguinolenta* obtained from the Supply Department of the Marine Biological Laboratory. A few specimens of *Asterias vulgaris* were also studied, but as these did not appear to react differently from *A. forbesi* further work on this species was not continued. All the animals used were about 2 inches in diameter. They were placed individually in beakers containing a medium consisting of 50 ml. of filtered sea water and dissolved, C^{14}-labeled nutrients. The specimens were left in this medium for a period of 8 hours (except those sacrificed at 1 hour), and then rinsed twice and placed in a holding tank of running sea water. While retained in the holding tank they were provided with a number of small clams to serve as food. The distribution of radioactivity in the tissues of groups of animals was analyzed following periods of 1, 8, and 72 hours, and 20 days, measured from the time the animals were first placed in the medium.

Two types of medium were used. One consisted of 0.5 microcurie (0.0033 mg.) of a mixture of 15 uniformly C^{14}-labeled, purified amino acids per 50 ml. of filtered sea water. The manufacturer of the amino acid mixture (New England Nuclear Corp. of Boston, Mass.) claims that it contains the "same relative proportions as found in a typical algal protein hydrolysate." The other medium consisted of 0.5 microcurie (1.85 mg.) of uniformly labeled C^{14}-glucose in each 50-ml. portion.

In order to measure the distribution of the labeled nutrients in the animals, each specimen was dissected as follows: the rays were cut off as near to the disk as possible. Incisions were then made up the lateral edges of each ray so that the oral and aboral portions could be separated. Next, the digestive glands were pulled free from the aboral portion. The disk was then picked up and each of its supporting columns severed so that it could be opened and the stomach (both cardiac and pyloric divisions) cut free.

As a result of this procedure five groups of tissue were obtained. These will be referred to as the "disk," "oral body wall," "aboral body wall," "stomach," and "digestive glands." The gonads were always included with the disk group, as their state of development was not consistent enough to warrant a separate set of analyses. Furthermore, preliminary studies had demonstrated negligible uptake of the nutrients by these structures.

The groups of tissues were then processed in two different ways for analysis of their radioactivity. The first method was designed to measure the total amount of material actually absorbed and retained. In it, the tissues were digested at 100° C. in test tubes with 1 ml. of 1 M NaOH in sea water. Digestion was enhanced by adding 1 or 2 drops of 30% H_2O_2. When all of the soft tissues were uniformly dispersed, the contents of the tubes were decanted into tared, 1-inch, stainless steel sample pans and dried in an oven. The radioactivity of each sample was measured in a Nuclear-Chicago, low-background, G-M counter fitted with a "Micromil" window. Corrections were made on the basis of infinite thickness and the counts compared to those of similarly prepared tissues to which known quantities of labeled nutrients had been added. The corrected measurements of 27 such standard samples had a mean deviation of 11.6%. The alkaline digestion was used in preference to solubilizing in acid as it prevented the loss of carbonaceous endoskeletal material.

The second method was intended to determine the amounts of absorbed nutrient materials which were retained by the tissues in a relatively unbound state. In this procedure, each group of tissue was extracted 48 hours in 10 ml. of ethanol solution. Based on the results of test runs, an 80% concentration of alcohol was found most satisfactory for the amino acid samples and a 40% solution best for the samples containing glucose. In both cases, duplicate 0.25-ml. aliquots of the extracts were plated onto $1\frac{1}{4}$-inch stainless steel sample pans, dried, and counted. Again, the counts were compared to those of samples to which known quantities of tracers had been added. The counts of 30 standard samples exhibited a mean deviation of 6.7%.

Two to 5 specimens of each starfish species were treated by both methods for each of the 8 different combinations of time interval and type of medium employed.

RESULTS

Quantity of nutrients taken up

Almost all the animals used in the study absorbed significant amounts of the labeled nutrients made available to them. In the experiments involving the amino

TABLE I

Distribution of exogenous amino acids taken up by starfish tissues.
(*Expressed as % of initial quantity to which animals were exposed*)

Time	Species	Disk		Oral body wall		Aboral body wall		Stomach		Digestive gland		Total	
		Wet wt. gm.	%	Wet wt. gm.	%	Wet wt. gm.	%	Wet wt. gm.	%	Wet wt. gm.	%	Wet wt. gm.	%
1 hour	*H. sang.*	1.85	8.1	1.54	19.0	0.72	10.8	0.23	0.1	0.47	0.2	4.81	38.2
	H. sang.	0.69	4.3	1.40	27.2	0.63	8.5	0.11	0.1	0.32	0.1	3.15	40.2
	A. forb.	0.70	11.2	1.26	25.0	0.67	26.8	0.06	0.1	0.40	0.1	3.09	63.2
	A. forb.	1.35	13.7	2.89	34.0	1.79	10.1	0.08	0.0	0.48	0.1	6.59	57.9
8 hours	*H. sang.*	0.51	10.3	1.37	27.1	0.76	11.7	0.07	0.1	0.17	0.1	2.88	49.3
	H. sang.	1.16	10.3	2.91	21.0	1.17	17.9	0.20	0.2	1.41	0.4	6.85	49.8
	A. forb.	0.79	6.7	1.12	21.4	0.56	9.6	0.13	0.1	0.36	0.2	2.96	38.0
	A. forb.	1.01	9.1	1.57	18.7	0.74	10.7	0.22	0.1	0.49	0.1	4.03	38.7
72 hours	*H. sang.*	1.01	11.5	1.41	32.3	0.59	15.9	0.46	0.2	0.70	0.5	4.17	60.4
	H. sang.	0.95	14.6	1.44	37.5	1.53	11.7	0.27	1.6	0.35	1.9	4.54	67.3
	A. forb.	1.18	9.0	3.15	23.3	2.67	17.4	0.11	0.1	0.80	0.7	7.91	50.5
	A. forb.	1.52	13.4	4.15	22.0	1.80	20.2	0.35	0.1	1.62	0.9	8.44	56.6
20 days	*H. sang.*	0.46	9.3	0.49	25.2	0.24	14.2	0.11	0.3	0.11	0.5	1.41	49.5
	H. sang.	0.86	11.0	0.69	25.2	0.36	27.4	0.17	0.2	0.22	0.4	2.30	64.2
	H. sang.	0.69	12.7	0.85	27.3	0.43	20.6	0.11	0.3	—	0.5	2.08+	61.4
	A. forb.	0.72	16.0	1.48	27.7	0.64	21.1	0.09	0.1	0.44	0.4	3.37	65.3
	A. forb.	0.77	12.9	0.48	35.1	0.77	14.5	0.08	0.1	0.40	0.4	2.50	63.0
	A. forb.	0.69	17.2	1.41	25.8	0.81	18.0	0.05	0.1	0.24	0.4	3.20	61.5

TABLE II

Distribution of exogenous glucose taken up by starfish tissues.
(Expressed as % of initial quantity to which animals were exposed)

Time	Species	Disk		Oral body wall		Aboral body wall		Stomach		Digestive gland		Total	
		Wet wt. gm.	%	Wet wt. gm.	%	Wet wt. gm.	%	Wet wt. gm.	%	Wet wt. gm.	%	Wet wt. gm.	%
1 hour	*H. sang.*	0.86	0.2	1.22	0.4	0.51	0.2	0.14	0.0	0.34	0.0	3.07	0.8
	H. sang.	1.10	0.3	1.49	0.7	0.58	0.4	0.24	0.0	0.33	0.1	3.74	1.5
	A. forb.	0.55	1.2	1.16	3.2	0.69	0.8	0.13	0.6	0.57	0.1	3.10	5.9
	A. forb.	1.14	2.1	2.04	5.7	1.19	2.1	0.21	0.0	1.36	0.1	5.94	10.0
8 hours	*H. sang.**	1.04	7.8	1.42	3.5	0.81	6.6	0.20	6.5	0.38	14.5	3.85	38.9
	*H. sang.**	0.96	7.6	1.47	2.5	0.71	3.1	0.23	13.9	0.46	38.6	3.83	65.7
	A. forb.	1.11	8.5	2.60	27.3	2.09	13.7	0.09	0.7	0.58	0.2	6.47	50.4
	A. forb.	1.34	7.1	3.16	27.9	2.21	8.8	0.18	0.1	1.07	0.2	7.96	44.1
72 hours	*H. sang.**	0.94	2.6	1.51	5.8	0.81	3.8	0.27	1.3	0.59	4.7	4.12	18.2
	H. sang.	1.15	1.2	1.92	7.3	0.97	2.7	0.16	0.1	0.55	0.1	4.75	11.4
	H. sang.	0.89	0.9	0.99	1.5	0.47	0.8	0.20	0.1	0.24	0.2	2.79	3.5
	*H. sang.**	0.77	0.9	1.30	1.5	0.84	1.7	0.10	0.6	0.31	1.2	3.32	5.9
	A. forb.	0.92	4.7	3.44	13.5	2.56	23.0	0.16	0.1	0.95	0.4	8.03	41.7
	A. forb.	0.88	9.1	1.97	34.4	1.21	6.9	0.10	0.1	0.67	0.2	4.83	50.7
20 days	*H. sang.**	0.73	1.6	0.80	1.3	0.41	0.6	0.08	1.4	0.12	1.1	2.14	6.0
	*H. sang.**	0.69	2.3	0.71	1.3	0.82	1.2	0.11	3.0	0.17	4.0	2.50	11.8
	H. sang.	0.63	0.6	0.61	1.1	0.32	0.5	0.11	0.1	0.13	0.2	1.80	2.5
	*H. sang.**	0.22	0.8	0.34	0.8	0.22	0.6	0.03	1.8	0.05	2.2	0.86	6.2
	H. sang.	0.32	0.3	0.38	0.6	0.21	0.5	0.05	0.1	0.10	0.1	1.06	1.6
	A. forb.	0.46	3.4	1.23	10.5	0.89	2.9	0.08	0.1	0.30	0.1	2.96	17.0
	A. forb.	0.95	6.6	1.84	25.8	0.98	6.8	0.16	0.3	0.74	0.3	4.67	39.8
	A. forb.	0.68	4.0	1.85	12.5	1.38	5.2	0.04	0.1	0.46	0.1	4.41	21.9

acid mixture (Table I), usually about 40 to 65% of the radioactive elements initially present was removed. Interestingly, most of this uptake appeared to take place during the first hour of incubation. In fact, with *Asterias,* the mean total values for absorption were less after 8 hours than they were after 1 hour (Fig. 1). Considering the variation between the different specimens, however, this apparent decrease probably would not have been observed if a larger number of animals had been tested.

Nevertheless, a very large proportion of the total uptake of the amino acids did take place with both species early in the incubation period. While the causes of this effect are uncertain, the property could have been due to at least two factors. Firstly, certain of the types of the amino acids included in the mixture presumably are more easily absorbed than others, and thus, these types would become rapidly depleted from the medium. The less-easily absorbed amino acids remaining after the first hour would be taken up more slowly over a longer period of time. Secondly, the organism could release substances which would accumulate in the sea water and, after an interval, some of these might reach concentrations sufficient to inhibit the absorption of the amino acids which had not yet been taken up. Such an inhibition would be relatively easy to achieve considering the small quantities of labeled amino acid used. Both of these phenomena have been observed in previous experiments

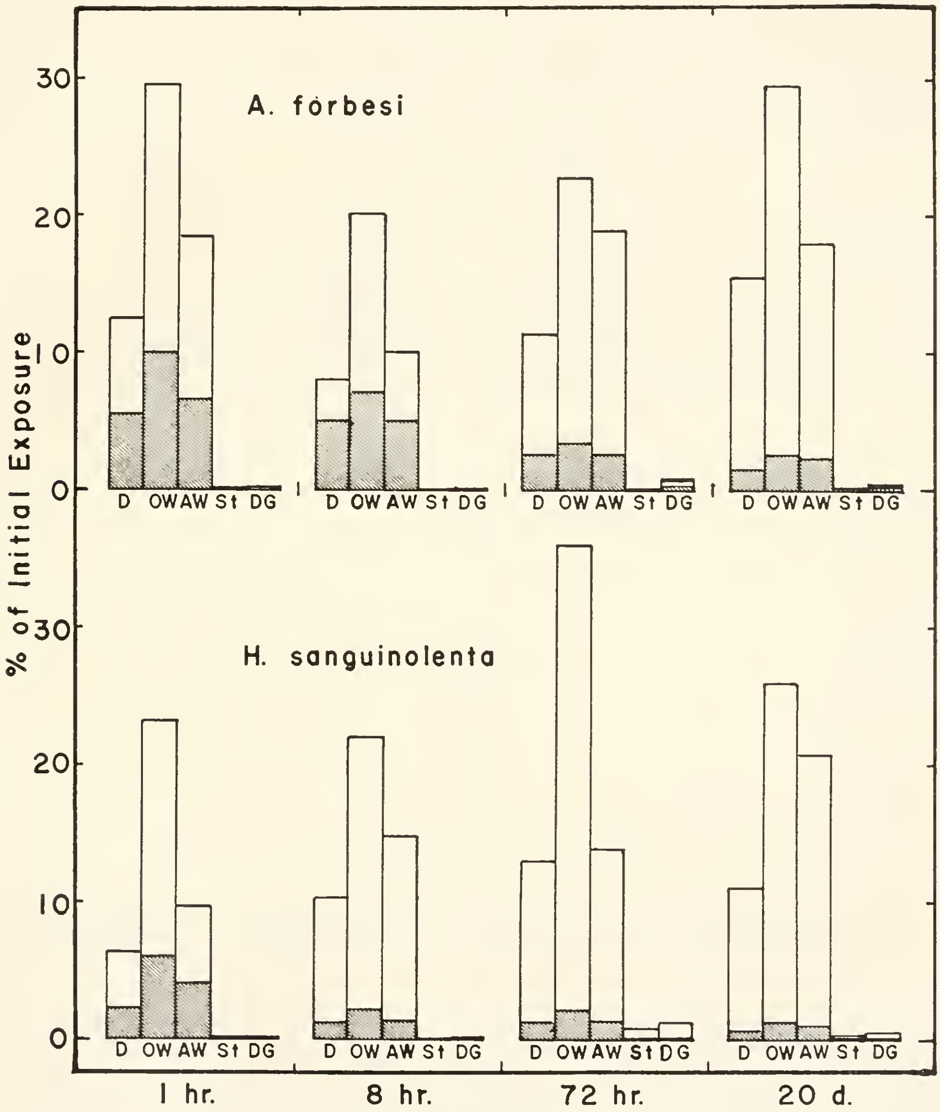

FIGURE 1. Quantities of absorbed amino acids found in five different body regions of specimens of two species of starfishes. Values (% of initial exposure) refer to the percentages of the total initial C^{14}-labeled amino acid present in the medium which were recovered from the different groups of tissues (mean 2–3 specimens). The entire bars represent the total uptake (digest method) while the cross-hatched areas represent material remaining unbound (alcohol extract method). D, disk; OW, oral body wall; AW, aboral body wall; St, stomach; DG, digestive glands. Over the 20-day period there is little redistribution of the absorbed amino acids. For further explanation, see text.

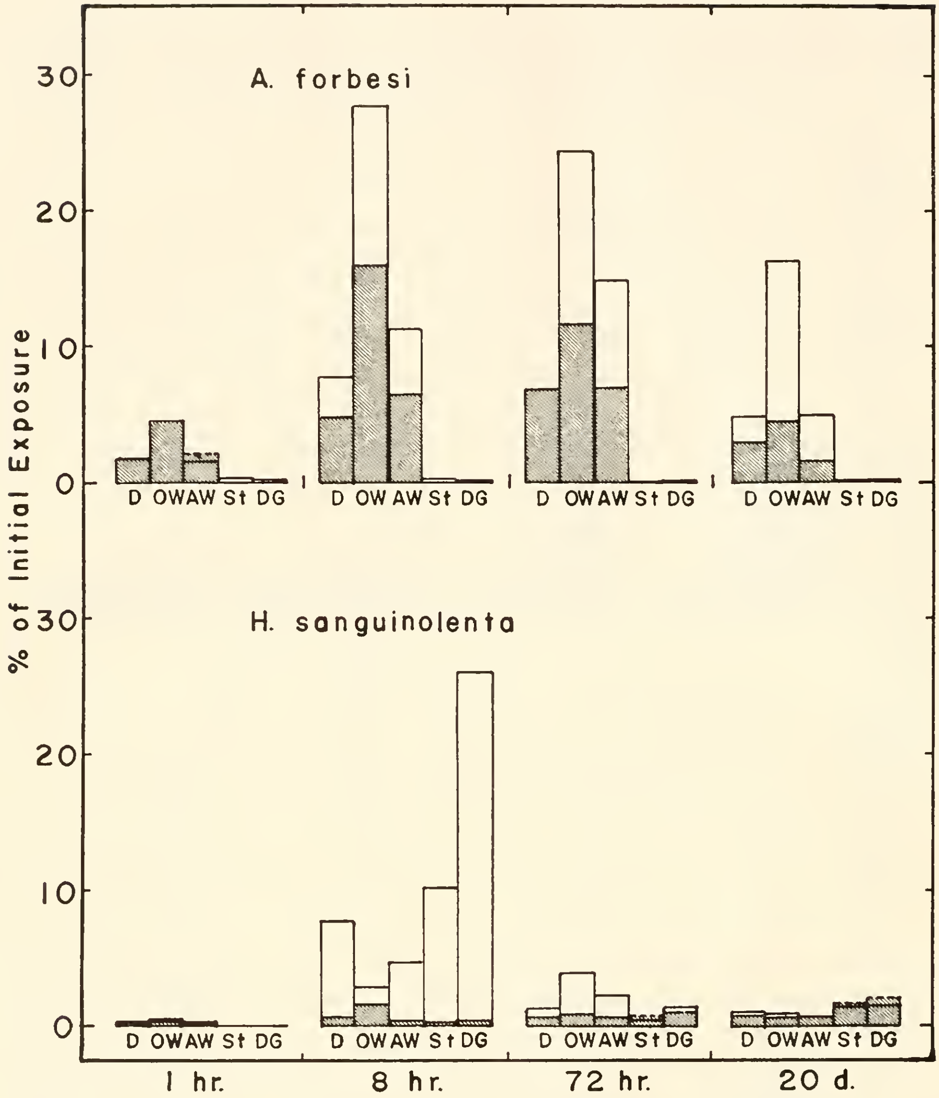

FIGURE 2. Quantities of absorbed glucose found in five different body regions of specimens of two species of starfishes. Values (% of initial exposure) refer to the percentages of the total initial C^{14}-labeled glucose present in the medium which were recovered from the different groups of tissues (mean 2–5 specimens). Symbols are the same as in Figure 1. Some specimens of *Henricia* apparently have taken up the glucose directly into their digestive organs. For further explanation, see text.

dealing with the uptake of amino acids by isolated starfish organs (Ferguson, 1964, and unpublished data).

In contrast to the time course of amino acid uptake which was observed, glucose apparently was absorbed continually over the 8-hour incubation period. This feature can be seen in Table II where the values for total per cent uptake by the 8-hour specimens are many times those of the 1-hour specimens. The glucose solution, unlike the amino acid mixture, was homogeneous. Also, the molar concentration of the glucose was considerably higher than that of the amino acids (because of its lower specific activity). Thus, while the percentages of glucose taken up appear to be somewhat lower than those of the amino acids, the actual quantities were probably much greater. Likewise, at the end of the incubation period the concentration of glucose still remaining in the medium was greater than even the initial concentration of amino acid used.

Distribution of the absorbed nutrients

With a few specific exceptions, practically all of the labeled nutrients which were taken up from the two types of medium were absorbed by the body wall components of the starfishes (Figs. 1 and 2). Very little (less than 1%) normally found its way into the internal organs. Even after 20 days there generally was no increase in the radioactivity of these structures which could be considered significant. The greatest quantities of the nutrients were most often found in the oral portions of the body wall. These substances were probably absorbed by the extensive surface of the tube feet and other areas of the epidermis of this region.

The mean values for the distribution of the glucose absorbed by *Henricia* (Fig. 2) present a pattern markedly different from that observed in the other cases. Indeed, in looking at the 8-hour specimens, the distribution is seen to be almost completely reversed; the least activity is found in the oral body wall and the greatest in the digestive glands. A study of the actual data which were recorded (Table II) helps to clarify what has happened. A number of the specimens of *Henricia* (marked *) show large values for the percentages of material taken up into their internal organs and low ones for the uptake into external parts. Other individuals of the species exhibit the opposite distribution and in this sense more closely resemble the specimens of *Asterias*. It appears, then, that the marked specimens responded to some stimulus, probably the relatively high glucose concentrations employed, by initiating a kind of feeding reaction in which the dissolved nutrient was removed from the medium by the internal digestive organs. The same phenomenon can also be noted in the data for some of the specimens which were extracted with alcohol (Table IV), but since the values recorded from these analyses are quite a bit lower, the differences do not stand out as pronouncedly.

Loss of nutrients taken up

After the completion of the 8-hour incubation period, there was little change in the total amino acid radioactivity observed in the various specimens (Fig. 1, Table I). Apparently, the tissues had a strong affinity for the amino acids once they had taken them up, and over the 20-day period did not release them back into the sea water or lose them through metabolism and respiration to any significant degree.

There was, however, a very marked loss of radioactive glucose from animals over the same period (Fig. 2, Table II). This reduction was most obvious in *Henricia*, but clearly also took place in the specimens of *Asterias*. While no evidence was obtained relative to the fate of this lost material, it most probably disappeared through the breakdown of the sugar by the cells and its release as respiratory CO_2.

Utilization of the absorbed nutrients

The analyses of the alcoholic extracts of the experimental animals provide data (Tables III and IV) through which additional insight may be gained into the ways in which the absorbed nutrients are utilized. This method measures only the labeled material which remains in a relatively "unbound" state after it is taken up.

TABLE III

Distribution of exogenous amino acids taken up by starfish tissues and retained in an unbound (alcohol-soluble) state. (Expressed as % of initial quantity to which animals were exposed)

Time	Species	Disk		Oral body wall		Aboral body wall		Stomach		Digestive gland		Total	
		Wet wt. gm.	%	Wet wt. gm.	%	Wet wt. gm.	%	Wet wt. gm.	%	Wet wt. gm.	%	Wet wt. gm.	%
1 hour	*H. sang.*	1.08	2.5	1.48	6.5	0.54	6.3	0.17	9.2	0.40	0.4	3.67	15.9
	H. sang.	0.82	1.9	1.18	5.6	0.54	1.8	0.09	0.1	0.16	0.1	2.79	9.5
	A. forb.	0.51	6.5	1.06	10.9	0.52	6.0	0.14	0.1	0.39	0.1	2.62	23.6
	A. forb.	1.03	4.6	1.94	8.8	1.74	7.0	0.04	0.1	0.68	0.0	5.43	20.5
8 hours	*H. sang.*	1.06	1.6	1.20	4.7	0.65	2.8	0.11	0.1	0.41	0.2	3.43	9.4
	H. sang.	1.24	1.6	2.53	3.4	1.23	2.6	0.71	0.1	0.12	0.1	5.83	7.8
	A. forb.	1.88	4.7	3.90	7.4	3.58	4.4	0.10	0.1	0.92	0.4	10.38	17.0
	A. forb.	3.21	5.4	5.10	6.6	3.29	5.6	0.29	0.2	0.99	0.0	12.88	17.8
72 hours	*H. sang.*	0.81	1.2	1.20	2.6	0.47	1.0	0.47	1.0	0.34	0.1	3.29	5.9
	H. sang.	1.36	1.3	1.49	1.7	0.70	1.9	0.16	0.2	0.27	0.1	3.98	5.2
	A. forb.	0.73	2.7	1.42	4.0	0.69	3.0	0.12	0.1	0.36	0.2	3.32	10.0
	A. forb.	2.51	2.4	3.30	2.9	1.64	2.3	0.33	0.0	1.04	0.1	8.82	7.7
20 days	*H. sang.*	0.43	0.7	0.78	1.5	0.30	0.9	—	0.2	0.01	0.2	1.52+	3.5
	H. sang.	0.36	0.6	0.42	1.2	0.18	0.9	—	0.1	0.01	0.1	0.97+	2.9
	A. forb.	1.10	1.5	1.67	2.1	1.23	2.5	0.16	0.2	1.07	0.3	5.23	6.6
	A. forb.	1.29	1.4	2.53	2.9	1.59	1.9	0.15	0.2	0.80	0.2	6.36	6.6

By comparison of these data with the results of the digestive method, an estimate can be obtained of the relative proportion of bound and unbound material retained by the cells at each period. These differences can be appreciated most easily with the aid of the two figures, by comparing the dark areas of each bar with the total length of the bar.

By such means it can be seen that, except for the initial periods, only a fraction of the material taken up normally was recoverable in the extracts. In the case of the amino acids (Fig. 1), the size of the soluble fraction decreased progressively in *Asterias* over the 20-day interval, from a peak at the end of the incubation period of 45% of the total amount absorbed to a low of 10% after 20 days. In *Henricia,* the range was from 32% at 1 hour to 5% at 20 days. It is interesting that in both species over one-half of the absorbed amino acid was unextractable with alcohol after only a single hour of incubation. Apparently, some of the absorbed amino acid was bound up quite rapidly while the rest remained in a soluble pool in the cells and was incorporated into proteins or metabolized much more slowly. There could possibly be some exchange between the soluble pool and the bound state. If such an exchange does occur, it presumably would also prolong the apparent time required for the extractable fraction to diminish.

TABLE IV

Distribution of exogenous glucose taken up by starfish tissues and retained in an unbound (alcohol-soluble) state. (Expressed as % of initial quantity to which animals were exposed)

Time	Species	Disk		Oral body wall		Aboral body wall		Stomach		Digestive gland		Total	
		Wet wt. gm.	%	Wet wt. gm.	%	Wet wt. gm.	%	Wet wt. gm.	%	Wet wt. gm.	%	Wet wt. gm.	%
1 hour	*H. sang.*	0.70	0.1	1.49	0.4	0.59	0.2	—	0.0	0.27	0.0	3.05+	0.7
	H. sang.	1.50	0.1	2.02	0.3	0.87	0.2	0.16	0.0	0.41	0.0	4.96	0.6
	A. forb.	0.76	1.9	1.70	4.9	1.15	2.1	0.03	0.0	0.67	0.0	4.31	8.9
	A. forb.	1.61	1.5	3.98	4.1	2.78	2.1	0.17	0.1	1.27	0.0	9.81	7.8
8 hours	*H. sang.*	—	0.8	—	1.5	—	0.6	—	0.5	—	0.8	—	4.2
	H. sang.	—	0.7	—	1.8	—	1.3	—	0.1	—	0.1	—	4.0
	H. sang.	1.02	1.8	1.19	1.6	0.55	1.1	0.10	0.6	0.43	2.0	3.29	7.1
	H. sang.	1.92	0.5	1.84	1.0	1.11	0.8	0.22	0.2	0.73	0.2	5.82	2.7
	A. forb.	—	4.1	—	16.8	—	4.6	—	0.2	—	0.1	—	25.8
	A. forb.	—	5.7	—	15.1	—	8.5	—	0.0	—	0.1	—	29.4
72 hours	*H. sang.*	0.99	0.3	1.29	0.7	0.60	0.5	0.12	0.1	0.36	0.1	3.36	1.7
	H. sang.	1.66	1.3	2.02	1.3	0.79	0.9	0.29	2.6	0.50	3.2	5.26	9.3
	H. sang.	1.25	0.7	1.26	0.5	0.81	0.7	0.14	0.9	0.25	0.7	3.71	3.5
	H. sang.	1.20	0.5	1.59	1.1	0.91	0.9	0.21	0.1	0.36	0.3	4.27	2.9
	A. forb.	0.99	3.1	0.96	7.9	2.48	7.0	0.07	0.1	0.42	0.1	4.92	18.2
	A. forb.	1.86	8.4	3.34	15.8	1.79	7.0	0.16	0.1	0.93	0.2	8.08	31.5
20 days	*H. sang.*	0.91	0.6	1.24	0.4	0.54	0.3	0.23	0.7	0.36	0.6	3.28	2.6
	H. sang.	0.22	0.9	0.41	0.6	0.21	0.7	0.02	2.7	0.07	4.0	0.93	8.9
	A. forb.	0.95	3.4	2.17	6.1	1.48	3.4	0.06	0.2	0.65	0.3	5.31	13.4
	A. forb.	0.91	2.7	1.66	3.0	2.20	0.3	0.11	0.1	1.08	0.2	5.96	6.3

Glucose was handled quite differently by the cells than were the amino acids. By the end of the first hour essentially all the glucose taken up was still unbound (Fig. 2). After 8 hours nearly 60% of the total quantity absorbed remained extractable in *Asterias*. (The calculated values for *Henricia* are insignificant because of the great amount of individual variation resulting from the apparent feeding behavior exhibited by some of these specimens.) Whether or not more of the glucose became bound cannot be determined from the data, since in the 3- and 20-day specimens there was a progressive loss of radioactivity, practically all of which appeared to be from the unbound material. The progressive disappearance of the unbound glucose seems to suggest that this fraction was the first to be metabolized and lost as CO_2. Again, some exchanges possibly could have occurred between the two fractions.

DISCUSSION

These experiments complement earlier work on the utilization of exogenous nutrients by starfish and confirm that at least two very different species of these animals possess biochemical mechanisms which enable them to remove various types of amino acids and glucose from sea water. These mechanisms apparently are efficient in picking up nutrients from even very dilute solutions. The limits of effectiveness of the absorptive machinery, however, have not been determined. Neither has much evidence yet been gathered concerning its chemical and physical properties.

While absorption probably can occur over all areas of the body surface, the greatest activity takes place in the oral region. This locality doubtless has the largest area of free surface, and very likely is more exposed to circulation of water than the other parts. It includes the tube feet, that protrude into the medium, and the entire region of the ambulacral groove, which is probably efficiently ventilated by means of ciliary tracts. Such tracts have been described repeatedly in various species, including *A. forbesi* (Budington, 1942) and *H. sanguinolenta* (Anderson, 1960; Rasmussen, 1965).

The full significance of the epidermal absorptive process is still uncertain. If Pequignat (1966) is correct in his conclusion that epidermal digestion by skin glands is a common phenomenon in starfish, one would expect the organisms to possess adequate mechanisms for the absorption and utilization of the different kinds of products released by such action. While the present investigation has indicated that some types of amino acids and glucose may be taken up through the epidermis, it is still undetermined if all the myriad types of organic compounds which presumably would be released through such a digestive process could be handled. In fact, as mentioned previously, the pattern of uptake observed for the mixture of amino acids suggests that certain types, representing nearly a third of the mixture, may not be readily absorbed. Likewise, there is as yet no confirmation that carbohydrates other than glucose can be utilized. Further investigations are contemplated which will more fully evaluate the diversity of compounds which may be taken up by epidermal mechanisms.

During the 20-day period in which the animals were studied there was little, if any, indication that nutrients were passed on to the internal regions of the body from the absorptive sites on the body surface. Very small amounts of radioactivity were detected in the internal organs of a few of the test specimens after several days, but since little consistency was seen, this activity was probably due to unavoidable contamination of the separate samples. Also, a few specimens may have ingested some of the slime, mucus, and algae which accumulated on the walls of the holding tanks, and this material could have picked up a slight amount of radioactivity. In any case, as the values observed for the internal organs are too low to be credited with significance, it should probably be concluded that epidermal absorption functions almost solely for the benefit of the superficial tissues.

The apparent feeding reaction exhibited by some of the specimens of *H. sanguinolenta* in the glucose medium is most interesting. Anderson (1960) carefully studied the structure and function of the digestive organs of this species and concluded that its Tiedemann's pouches were a "hydrodynamic organ or flagellary pump of prodigious effectiveness" (p. 393). He showed that *Henricia* was primarily a filter-feeder and could take up and entrap such material as suspended *Mytilus* sperm. Feeding experiments were also performed on *Henricia* by Rasmussen (1965). These were more quantitative than Anderson's and served to confirm further the great efficiency of this animal as a particle-suspension feeder.

The present observations reveal that the flagellary feeding mechanism described by the above workers can also be effective in the utilization of dissolved nutrient materials of relatively low molecular weight. The pumping mechanism of *Henricia* is apparently so efficient that it "pays" the animal to take up solutions of nutrients, provided they occur in at least minimal concentrations. The most significant aspect of these observations, however, is not so much the uptake of the dissolved materials, but rather, the nature of the stimulus which caused them to be taken up. Although further verification is needed, the stimulus appears to have been the relatively higher concentration (when compared to that of the amino acids) of the glucose solution used. This was the only variable observed in the experiments other than the type of compounds themselves.

Doubtless, in nature these animals frequently encounter various kinds of dissolved nutrients in equivalent or even greater concentrations than those used in the experiments. Some of these probably come from the external digestion of relatively solid organic substrates. The stomach of *Henricia* is rather unique among starfishes in possessing numerous zymogen cells (*cf.* Anderson, 1960), which likely are a source of enzymes for such a process. In the present experiments specimens were often seen in an apparent feeding position, with their stomachs everted as button-like protuberances applied against the algae-covered aquarium wall or between the valves of a gaping clam. Under normal circumstances, digestive products released during this activity would probably set off the pumping process. As the glucose in the experiments seems to have elicited the same response as the natural stimulus, one can conjecture that encounter by the animal of a significant concentration of dissolved nutrients in its environment could serve also as an effective stimulus for initiating the pumping process. Once pumping is started, the soluble nutrients are efficiently taken up into the internal digestive organs.

Henricia, then, seemingly obtains its nutrition through several different processes. It depends primarily on the suspended and dissolved materials normally present in the environment, but probably also can digest some solid food outside of its body. These nutritional substances apparently are taken up by means of flagellary currents, and absorbed internally, or, at least in part, are directly assimilated by the superficial tissues of the body which are also exposed to the substances.

An uptake of labeled glucose into the digestive system of *Asterias,* comparable to that observed in *Henricia,* was not noted. This difference in behavior probably was due to the fact that *Asterias* is primarily a predator. While it lacks the complex pumping apparatus possessed by *Henricia,* it does possess ciliated surfaces on its stomach. It relies on currents produced on these surfaces to bring in concentrated solutions of nutrients from victims digested externally by enzymes supplied from the digestive glands *via* gutters in the stomach wall (*cf.*, Anderson, 1954). This process is probably not altogether different from the pumping of *Henricia*. In a previous note (Ferguson, 1963b), for example, I reported the uptake into the digestive organs of *Asterias* of C^{14}-labeled glucose and amino acids which had been injected into small clams just before they were fed to the starfish. In that case, the presence of the solid pieces of food appears to have stimulated the animals to activate their feeding mechanism. As in *Henricia*, once feeding was initiated, uptake of the dissolved materials into the digestive organs proceeded rapidly.

The probability that epidermal absorption of exogenous nutrients is a continuous process while normal feeding is generally a discontinuous one is perhaps quite significant. In a sense, the two activities may balance each other as sources of nutrition over a period of time. If such is the case, the internal regions of the body may be seen as receiving nutrition almost exclusively *via* the digestive tract, while the more external tissues would be nourished to a considerable extent directly through the epidermis. One might suppose, then, that if an animal were prevented from utilizing either one of the sources, it probably could not survive. In this vein, investigations have shown that various species of starfishes can live long periods with little or no visible food, but they cannot subsist indefinitely under such conditions (Galtsoff and Loosanoff, 1939; Vevers, 1949). It would be much more difficult to design an experiment in which specimens were allowed to eat but completely denied epidermal absorption. But since it has been determined that epidermal absorption of nutritional materials does occur, and can take place to a significant degree, it seems reasonable to conclude that such absorption is an important factor in the economy of these organisms.

SUMMARY

1. Small specimens of *A. forbesi* and *H. sanguinolenta* were exposed to dissolved C^{14}-amino acids and glucose. The subsequent distribution of these materials was then determined in the following five regions of the body: disk (including the gonads), oral body wall of the rays, aboral body wall of the rays, stomach, and digestive glands.

2. In all cases, large proportions of the labeled nutrients were taken up into the external tissues. The largest amount was usually absorbed into the oral body wall, which probably possesses a proportionately greater ventilated surface area than the other regions.

3. Over a period of 20 days there was little indication of movement of the externally absorbed nutrients into the internal organs. In this period, very little loss of amino acid radioactivity was noted. The amino acids became progressively less soluable in alcohol, suggesting that they were incorporated into the structural proteins of the organism.

4. Glucose radioactivity declined progressively over the 20-day period. As observed in *Asterias*, this decline occurred almost exclusively in the portion of absorbed glucose that remained alcohol-soluble. This fraction was possibly used as an energy source while the insoluble fraction became incorporated into more inert elements.

5. A number of the specimens of *Henricia* appeared to pump up and absorb the glucose medium into their digestive organs. This was interpreted as a form of

feeding behavior possibly initiated by the relatively high concentration of glucose used. The much less concentrated amino acid medium failed to initiate such a reaction.

6. It is concluded that nutrition in starfish is probably a dual process involving both a continuous epidermal absorption of dissolved exogenous materials for the benefit primarily of the superficial tissues, and intermittent oral feeding to satisfy the more general needs of the entire organism and especially of the internal organs.

LITERATURE CITED

Anderson, J. M., 1954. Studies on the cardiac stomach of the starfish, *Asterias forbesi*. *Biol. Bull.*, **107**: 157–173.

Anderson, J. M., 1960. Histological studies on the digestive system of a starfish, *Henricia*, with notes on Tiedemann's pouches in starfishes. *Biol. Bull.*, **119**: 371–398.

Budington, R. A., 1942. The ciliary transport-system of *Asterias forbesi*. *Biol. Bull.*, **83**: 438–450.

Ferguson, J. C., 1963a. The physiological mechanisms of nutrient transport in the starfish, *Asterias forbesi*. Doctoral dissertation, Cornell University.

Ferguson, J. C., 1963b. An autoradiographic study of the distribution of ingested nutrients in the starfish, *Asterias forbesi*. *Amer. Zool.*, **3**: 524.

Ferguson, J. C., 1964. Nutrient transport in starfish. II. Uptake of nutrients by isolated organs. *Biol. Bull.*, **126**: 391–406.

Galtsoff, P. S., and V. L. Loosanoff, 1939. Natural history and method of controlling the starfish (*Asterias forbesi*, Desor). *Bull. U. S. Bureau Fisheries*, **49**: 75–132.

Pequignat, E., 1966. Skin digestion and epidermal absorption in irregular and regular urchins and their probable relation to the outflow of spherulecoelomocytes. *Nature*, **210**: 397–399.

Pütter, A., 1909. Die Ernährung der Wassertiere und der Stoffhaushalt der Gewässer. Fisher, Jena.

Rasmussen, B. N., 1965. On taxonomy and biology of the North Atlantic species of the asteroid genus *Henricia* Gray. *Meddelelser fra Danmarks Fiskeri- og Havundersøgelser*, **4**: 157–213.

Stephens, G. C., 1962. Uptake of organic material by aquatic invertebrates. I. Uptake of glucose by the solitary coral, *Fungia scutaria*. *Biol. Bull.*, **123**: 648–659.

Stephens, G. C., 1963. Uptake of organic material by aquatic invertebrates. II. Accumulation of amino acids by the bamboo worm, *Clymenella torquata*. *Comp. Biochem. Physiol.*, **10**: 191–209.

Stephens, G. C., 1964. Uptake of organic material by aquatic invertebrates. III. Uptake of glycine by brackish-water annelids. *Biol. Bull.*, **126**: 150–162.

Stephens, G. C., and R. A. Schinske, 1961. Uptake of amino acids by marine invertebrates. *Limnol. and Oceanog.*, **6**: 175–181.

Stephens, G. C., and R. A. Virkar, 1965. Accumulation and assimilation of amino acids by the brittle star, *Ophiactis simplex*. *Amer. Zool.*, **5**: 661.

Stephens, G. C., and R. A. Virkar, 1966. Uptake of organic material by aquatic invertebrates. IV. The influence of salinity on the uptake of amino acids by the brittle star, *Ophiactis arenosa*. *Biol. Bull.*, **131**: 172–185.

Vevers, H. G., 1949. The biology of *Asterias rubens* L.: growth and reproduction. *J. Mar. Biol. Assoc.*, **28**: 165–187.

ENVIRONMENTALLY CONTROLLED INDUCTION OF PRIMARY MALE GONOCHORISTS FROM EGGS OF THE SELF-FERTILIZING HERMAPHRODITIC FISH, RIVULUS MARMORATUS POEY

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The fact of genetic sex determination among teleosts is well established by sex linkage (Gordon, 1957), although cytological demonstrations of fish sex chromosomes have not withstood critical scrutiny (White, 1954) until recently (Nogusa, 1960). Both male and female fish with phenotypic sex contrary to genotypic sex have been produced by early treatment with sex steroids (Yamamoto, 1953–1961). Can genetic sex determination in fishes be overridden also by external environmental factors, as in some amphibians, is the question to which answers were sought in the experiments to be reported here.

This question was first raised by effects on anuran sex determination of delayed fertilization (overripeness of eggs) and of temperature obtained, respectively, by Pflüger (1882) and Witschi (1929). Comparable experiments on fishes have been few; their long duration with no assurance of negotiable results discourages investigation. Under harsh contrasting experimental conditions it is extremely difficult to rear fish through the early crises of ontogeny without excessive losses, and if mortalities exceed a certain limit, the dilemma of a differential mortality of one sex *versus* experimental induction of the other cannot be resolved.

Conclusive evidence of environmental influence on sex determination in teleosts is lacking despite possible indications of such influence from experiments on one species each of the genera *Salmo* (Mršić, 1923), *Betta* (Eberhardt, 1943), and *Anguilla* (D'Ancona, 1950, 1960). Only by making explicit certain crucial defects in these experiments passed over by reviewers can the rationale of our own experiments and the cogency and singularity of their results be given their full context (see Discussion).

The cyprinodontid fish used in the present study, *Rivulus marmoratus* Poey, is unique among fishes so far as known in being comprised of natural, consistently self-fertilizing hermaphrodites (Harrington, 1961, 1963; comments of Atz, 1964). Its hermaphroditism is normal and not a laboratory artifact like that of *Lebistes reticulatus* (Spurway, 1957), for example. Long deemed merely a nominal species (Garman, 1895), *R. marmoratus* was revived as a valid species by Rivas (1945), who rediscovered its types in the U. S. National Museum, but was unknown as a living fish until it was found in Florida (Harrington and Rivas, 1958). Tissue grafts between Florida wild-caught progenitors and their laboratory-reared descendants gave the *autograft reaction* (Kallman and Harrington, 1964), indicating that they have the same genotype and that probably their wild antecedents also had reproduced by self-fertilization, *i.e.* for upwards of 10 generations (see below).

Although an allegedly gonochoristic subspecies, *R. marmoratus bonairensis,* was described from the Antilles (Hoedeman, 1958) the same year that we found *R. marmoratus* in Florida, we have no evidence from the laboratory or from the wild that females exist in Florida and so far have found no males in the wild. It came as a surprise, therefore, when males appeared among hermaphrodites propagated in our laboratory, especially since these were bright orange with the caudal ocellus obsolescent, in sharp contrast to the hermaphrodites. The incidence of males has stayed below 5% through more than 10 uniparental laboratory generations, numbering over 350 fish, each isolated throughout life to exclude physiological interactions of any kind except visual ones.

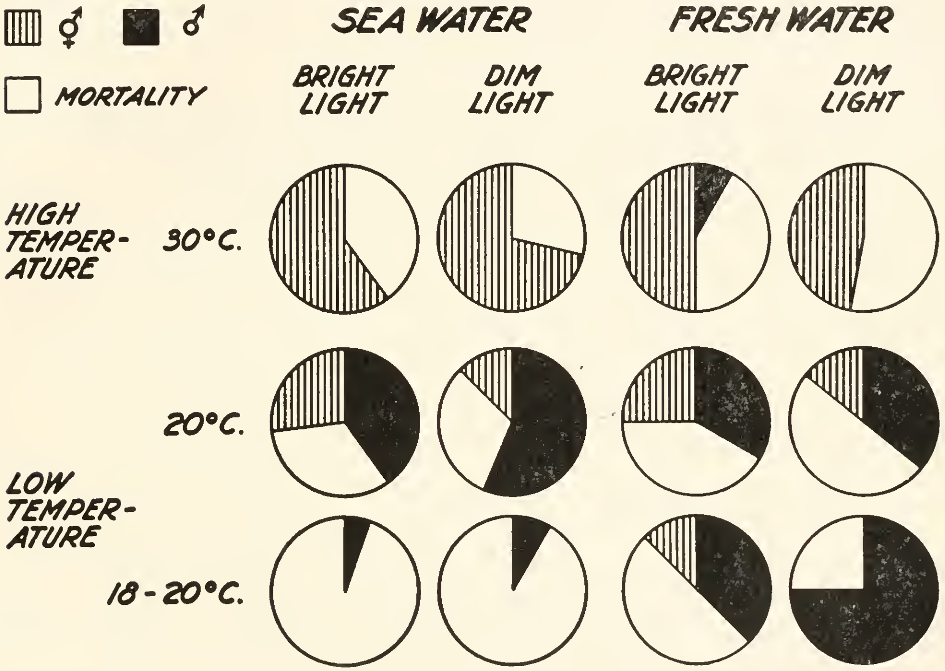

FIGURE 1. Plan and results of Experimental Series One, data in Table I. Individuals of *Rivulus marmoratus,* each in its own jar, were exposed to the eight combinations of bright or dim light, sea water or fresh water, high or low temperature. Exposure was from not later than the ¾ blastoderm stage until sexual maturity at high temperature or five months post-hatching at low. Circles show the percentages of hermaphrodites and males and the percentage mortality resulting from exposure to each extrinsic factor combination. The temperature of the lowermost row of treatments was raised to 20° C. when 18° C. proved lethal in combination with sea water, and the middle row, at 20° C., was added. Compare with the results of Experimental Series Two (Table IV).

The appearance of an occasional male in clones otherwise composed of hermaphrodites suggests some lability in the sex-determining mechanism through which the genotype normally produces the hermaphrodite phenotype. It seems proper to speak here of a hermaphrodite genotype, because as Atz (1964) observes, the assumption is false that *normal hermaphroditism* cannot be genetically controlled, as is the sex of gonochorists. The present experiments were contrived to identify a possible external environmental factor capable of causing a deviation to the male phenotype during sex differentiation. Positive results were obtained in two series of experiments, the first begun in August, 1961, the last completed in January, 1965.

MATERIALS AND METHODS

Few if any experiments on vertebrates can have used material as genetically uniform as the *Rivulus marmoratus* eggs used here. In Experimental Series One (Fig. 1 and Table I), the fish surviving to be sexed hatched from eggs of hermaphrodites of two clones, 32 Clone-NA eggs and 41 Clone-DS eggs (Table II). The fish of Table II are coded as they were when used in the graft tests providing the evidence for these clones (Kallman and Harrington, 1964). Contrary to two data (*loc. cit.,* Table III, #II and #IV), however, Fish DS, Fish FT, and Fish NSU all belong to the same clone, later interline grafts (unpublished) having given the autograft reaction, showing that the previous rejections (#II and #IV) were mechanical and not immunological. In Experimental Series Two (Table IV), all were Clone-DS eggs of Uniparental Laboratory Generations 9, 10, and 11, so that

TABLE I

Effects of external factors on the sex ratio of uniparental offspring of Rivulus marmoratus *hermaphrodites. Self-fertilized eggs were reared from outset of extraparental incubation under various combinations of light intensity, salinity and temperature.*
B, bright light; D, dim light; S, sea water; F, fresh water;
18/20°C., started at 18° C. but continued at 20° C.

Treatment		Surviving/ treated	Percentage survival	Survivors				Non-survivors					
				Hermaphrodites		Males		Died *in ovo* or at hatching		Died very small		Extremely abnormal; discarded	
				Total	%	Total	%	Total	%	Total	%	Total	%
30° C.	B S	6/10	60.0	6	100.0		0.0	4	100.0		0.0		0.0
	D S	5/7	71.4	5	100.0		0.0	1	50.0	1	50.0		0.0
	B F	7/12	58.3	6	85.7	1	14.3	2	40.0	3	60.0		0.0
	D F	8/17	47.1	8	100.0		0.0	6	66.7	1	11.1	2	22.2
20° C.	B S	10/15	66.7	4	40.0	6	60.0	4	80.0		0.0	1	20.0
	D S	11/16	68.8	2	18.2	9	81.8	3	60.0		0.0	2	40.0
	B F	7/12	58.3	3	42.9	4	57.1	1	20.0	2	40.0	2	40.0
	D F	7/14	50.0	2	28.6	5	71.4	2	28.6	5	74.1		0.0
18/20° C.	B S	1/19	5.3		0.0	1	100.0	16	88.9		0.0	2	11.1
	D S	1/12	8.3		0.0	1	100.0	5	45.4	3	27.3	3	27.3
	B F	4/8	50.0	1	25.0	3	75.0	1	25.0	1	25.0	2	50.0
	D F	6/8	75.0		0.0	6	100.0		0.0	2	100.0		0.0
30° C.	All	26/46	56.5	25	96.2	1	3.8	13	65.0	5	25.0	2	10.0
20° C.	All	35/57	61.4	11	31.4	24	68.6	10	45.5	7	31.8	5	22.7
18/20° C.	All	12/47	25.5	1	8.3	11	91.7	22	62.9	6	17.1	7	20.0

TABLE II

Sex ratios of progeny of self-fertilized Rivulus marmoratus *reared* ab ovo *from outset of extraparental incubation at either high or low temperature, showing the same temperature correlation regardless of parentage or clone.* Same data as in Table I*

Parent	Progeny surviving/treated	Percentage survival	High temperature (30° C.)				Low temperature (18–20° C.)			
			Hermaphrodites		Males		Hermaphrodites		Males	
			Total	%	Total	%	Total	%	Total	%
FT	19/42	45.2	5	100.0		0.0	2	14.3	12	85.7
NSU	2/2	100.0	1	100.0		0.0		0.0	1	100.0
DS P_1	6/11	54.5	3	100.0		0.0	1	33.3	2	66.7
F_1	6/28	21.4	1	100.0		0.0	3	60.0	2	40.0
F_2	8/14	57.1	1	100.0		0.0	4	57.1	3	42.9
NA	32/52	61.5	14	93.3	1	6.7	2	11.8	15	88.2
NSB	0/1	0.0								
Totals	73/150	48.7	25	92.6	1	3.8	12	25.5	35	74.5

* Wild-caught fish FT, NSU, and DS and their uniparental descendants belong to the same clone; wild-caught NA belongs to a different clone (Kallman and Harrington, 1964, and unpublished).

TABLE III

Sex ratios of progeny of self-fertilized Rivulus marmoratus *reared at either high or low temperature, showing the same temperature correlation regardless of developmental stage at outset of treatment (= outset of extraparental incubation). Same data as in Tables I–II.* Stage 1 is the fertilized egg before polar cap formation; at Stage 13c the blastoderm encloses ¾ of the yolk. For intervening stages see Harrington, 1963

Developmental stage at outset	Surviving/treated	Percentage survival	High temperature (30° C.)				Low temperature (19–20° C.)			
			Hermaphrodites		Males		Hermaphrodites		Males	
			Total	%	Total	%	Total	%	Total	%
1	0/1	0.0								
2	1/4	25.0						0.0	1	100.0
3	1/5	20.0						0.0	1	100.0
4	5/13	38.5	4	80.0	1	20.0				
5	8/16	50.0	3	100.0		0.0	2	40.0	3	60.0
6	16/21	76.2	4	100.0		0.0	1	8.2	11	91.8
7	5/15	33.3	2	100.0		0.0	1	33.3	2	67.0
8a	4/6	66.7	2	100.0		0.0		0.0	2	100.0
8b	5/10	50.0	2	100.0		0.0		0.0	3	100.0
8c	2/6	33.3					2	100.0		0.0
8d	9/16	56.3	2	100.0		0.0	2	28.6	5	71.4
9	5/17	29.4	1	100.0		0.0	3	75.0	1	25.0
10	6/9	66.7	2	100.0		0.0	1	25.0	3	75.0
11	0/1	0.0								
12b	3/4	75.0	2	100.0		0.0		0.0	1	100.0
13a	1/2	50.0	1	100.0		0.0				
13c	2/3	66.7						0.0	2	100.0
Totals	73/150	48.7	25	96.2	1	3.8	12	25.5	35	74.5

besides the immunological evidence that the fish at the outset of these generations were of one clone (Kallman and Harrington, 1964), selfing through eight generations alone would have brought them to over 99% homozygosity (Sinnott and Dunn, 1939; p. 284). Alternatively, in the remote contingency of a homozygote-preventing mechanism, they would share the same heterozygous genotype.

The wild-caught progenitors of Table II were isolated from date of capture. Every other fish referred to in this report was kept in lifelong isolation begun at its retrieval as a self-fertilized egg being emitted by its parent. Eggs of *R. marmoratus* are laid after *intraparental incubation* for from a few minutes to 2½ days, *viz.* from in Stage 1 (just fertilized) to in Stage 24 (prominent pectoral fin buds), as before described (Harrington, 1963). Eggs for our experiments were sucked into a pipette as they fell from laying hermaphrodites, kept at a water temperature of about 25° C. Developmental stages at outset of experimental treatment varied according to the experiment. Each egg of suitable stage was pipetted into its own jar. The egg in its own jar was put under the conditions of its allotted treatment, encompassing *extraparental incubation,* hatching and subsequent life in this jar, except for one low-temperature treatment (Table IV, B) begun with hatchlings.

TABLE IV

The sex determination and differentiation of uniparental Rivulus marmoratus *modified by temperature. Self-fertilized eggs from hermaphrodites of a single clone were reared under contrasting temperature regimes, but with light intensity and salinity controlled. Compare with Table I and Figure 1*

Temperature regime	Total eggs reared	Hermaphrodites		Males		Mortality	
		No.	%	No.	%	No.	%
A) 25 ± 1° C. to maturity (control)	50	50	100	0	0	0	0
B) 25 ± 1° C. through hatching; 19.5 ± 0.5° C. for the first 5 months post-hatching; 25 ± 1° C. thereafter to maturity	50	46	92	0	0	4	8
C) 25 ± 1° C. to at least Stage 16 but not beyond Stage 22a; then 19.5 ± 0.5° C. through eclosion* and for 5 months post-eclosion; thereafter 25 ± 1° C. to maturity	50	9	18	36	72	5	10

* Eclosion refers to either hatching or being cut out of the chorion.

Wide-mouthed, straight-sided, cylindrical, screw-top jars were used. These were about 15 cm. high and 8 cm. in diameter, holding 950 ml., and were filled with 600 ml. of water. Plastic Petri-dish covers used as lids prevented escape, without injury to jumping fish or interference with gas exchange. Fish have lived in these jars for 45 months, and as many as 500 were kept concurrently, each in its own jar, during our experiments. Unremitting care was taken to exclude any possibility of transfer between jars of physiological substances. A utensil inserted in one jar was rinsed repeatedly in a container overflowing with fast-running water before being inserted in another.

Jars going from propagating room (25° C.) to the low experimental temperature (constant-temperature room) were moved immersed in a water bath at 25° C. When the jar water reached the low temperature, the jars were taken out, wiped off, and left in the constant-temperature room. Jars going to the high experimental temperature were put in a water bath to raise the jar water to this high temperature. Then the jars were moved to the constant-temperature room and left immersed for the duration of the treatment in water baths thermostatically controlled to maintain the high experimental temperature. These procedures were reversed when fish were returned to the propagating room for post-treatment observation. The conditions of each experimental treatment were maintained continuously. As new eggs became available in temporal succession, each was allocated to one of the treatments singly and in its own jar. Each jar was removed from the treatment conditions and returned to the propagating room when its fish reached functional sexual maturity, or after five months in the case of low-temperature treatments.

The fish in jars returned to the propagating room for post-treatment observation were kept in 40% sea water for the remainder of their lifelong isolation. If not originally in 40% sea water, they were changed to it gradually over three days from their former (treatment) salinity. The propagating room received light within the natural daily photoperiod only, and the room temperature was constrained by an air conditioner and thermostatically controlled heater to hold the water temperature to about 25° C.

With the release of the hatchling from its egg *chorion* (terminology of Lord Rothschild, 1958), feeding was begun, first with microworms (nematodes), then with these mixed with brine shrimp (*Artemia*) nauplii. A premature diet of *Artemia* can cause death through intestinal stoppage, the shift to food of larger size evidently being a crisis of ontogeny. Afterwards, brine shrimp alone were used. Feeding was *ad libitum;* the amount squirted into the jar with a syringe was adjusted to fish size and to volume of unconsumed food in its jar each morning. Unless otherwise stated, food was introduced in water of the same salinity as in the jar. After feeding began, the jar water was filtered weekly, by pouring through filter paper on a glass funnel into a clean jar. The fish was transferred by syringe or net to the filtrate when it was deep enough. The old jar was washed, and filtrate and fish poured back into it. The new jar, the funnel, and the syringe or net were washed, and, with fresh filter paper, used for the next filtering. Cloudy water was replaced. New water replacing old or added to compensate for loss was of the same temperature and salinity. Jar water was kept a pale blue with methylene blue. Without this bacteriostatic dye eggs and larvae seemed to have a lower survival, but this was not tested experimentally. One or more times a day, solid wastes, uneaten food, and later on, eggs, if laid by the then mature fish, were sucked out with a syringe.

The jars were monitored daily, at first for hatchlings or eggs in Stage 31, which precedes hatching (Stage 32) under natural conditions, and for abnormal, sick and dead eggs or hatchlings, later for the first external signs of sex differentiation. Throughout the life of each hermaphrodite a daily record was kept of the number, conditions, and stages of eggs found in its jar. Under certain treatments, environmental cues normally triggering the hatching mechanism were either absent or nullified (*cf.* Kinne and Kinne, 1962), because hatching proved to be another crisis of ontogeny. Prolonged delays caused deaths through inanition (but see Harrington, 1959). In the first series of experiments, the mechanism could sometimes be activated by focusing bright light on overdue eggs, but it became the practice to cut from their chorions other embryos of the same age as those hatching naturally or with light stimulation (Table V). In the second series, the crisis was circumvented by cutting out all embryos incubated at low temperature (Table IV, C) long before the normal hatching stage, allowing the rest (Table IV, A and B) to hatch naturally, and mortalities did not exceed statistically permissible limits in the second series as they did in the first.

Hermaphrodites and males at the same temperature become externally recognizable as such at about the same age and size. Hermaphrodites retain the caudal ocellus possessed by both juvenile hermaphrodites and juvenile males, and acquire no orange pigmentation later. Sperm production in hermaphrodites is not copious enough to be visible as milt. The characteristic behavior pattern leading up to oviposition (Harrington, 1961) is confined to hermaphrodites. Each hermaphrodite was performance-proven in lifelong isolation by laying eggs from which normal fish hatched. Maturing males first acquire scattered, small orange spots on the body and minute orange flecks in increasing density on the fins. An orange wash later covers the whole body, as the caudal ocellus becomes obsolescent or vanishes altogether. Handled males often release milt, which in hanging-drop suspension shows active spermatozoa.

It is of fundamental importance to make clear that these are males *ab initio,* sometimes called "pure males," but more exactly, *primary male gonochorists.* This was established by serial sections of over 200 gonads from ontogenetic series of both hermaphrodites and males, ranging from germ cell entry into genital ridges to senility, as well as by contrast with testes of *secondary male gonochorists,* that may arise from older hermaphrodites by involution of the ovarian component of the ovotestes with proliferation of the testicular component, under conditions to be described in a separate report. Embryological and histological details are beyond the scope of the present report, but will be given elsewhere. A testis and an ovotestis, each in transverse section, and a male and hermaphrodite, both adults, are shown in Figure 2.

EXPERIMENTAL SERIES ONE

The treatments were the eight combinations of high *versus* low temperature, sea water *versus* fresh water, and bright *versus* dim light (Fig. 1 and Table I). Eggs ranged from the one-cell stage to Stage 13c (¾ blastoderm) at outset of treatment (Table III), which began within five minutes after the egg was emitted by its parent. The constant-temperature room thermostat was set at first to give a low water temperature of 18° C., but this proved lethal in combination with sea water (Fig. 1 and Table I). Survivors begun at 18° C. were continued at 20° C., and eggs of a new set were started at 20° C. These low water temperatures actually fluctuated between 20° C. and 21° C., being mostly closer to 20° C., and will be referred to as 20° C. for convenience. The high-temperature jars were immersed to the level of the water within them. They rested on a wire-screen platform above the bottom, their lids just clearing the underside of the glass aquarium covers. The bath water was circulated by air stones to equalize the temperature, held by thermo-

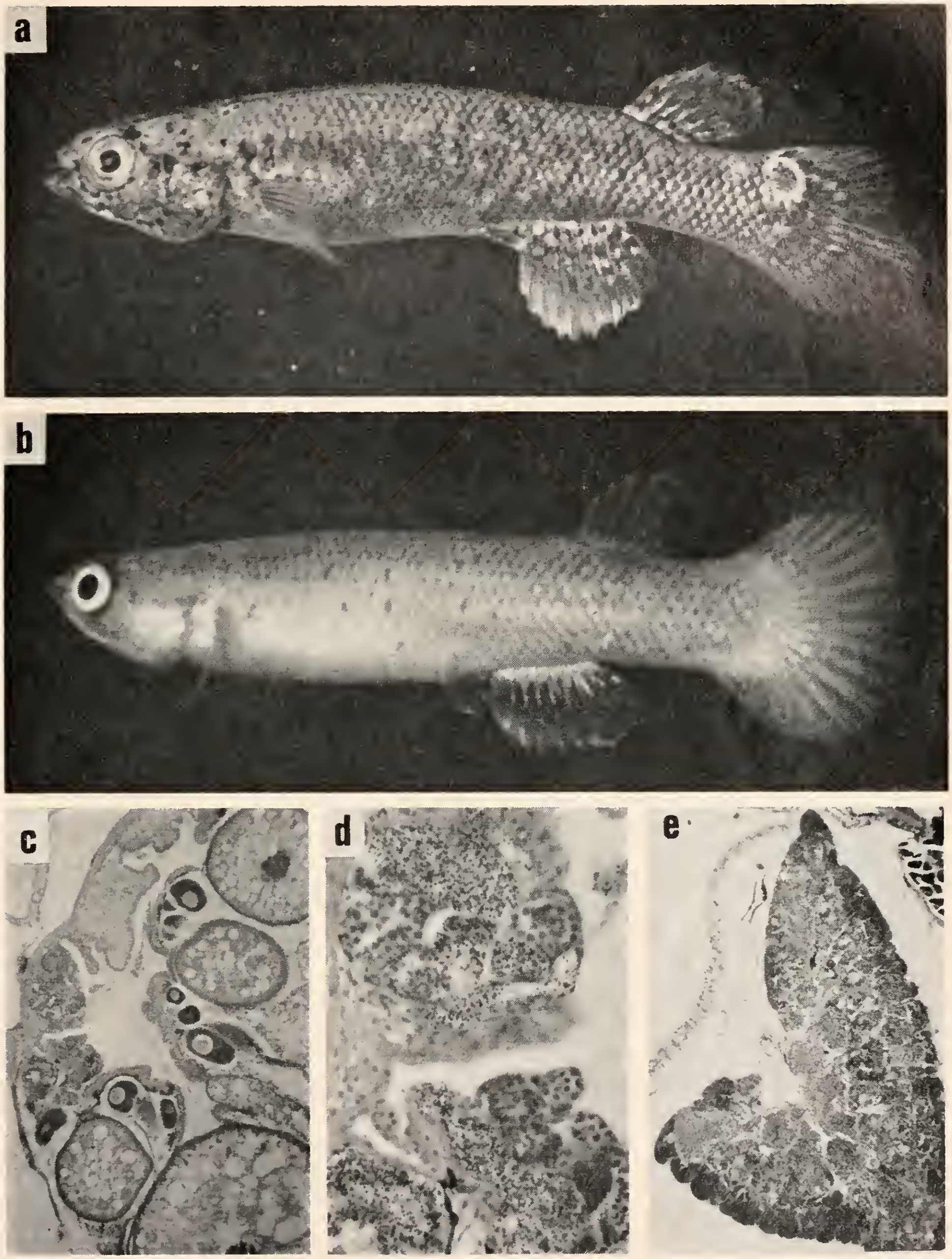

FIGURE 2. Young adult *Rivulus marmoratus.* a, hermaphrodite; b, primary male gonochorist; c, cross section of right lobe of ovotestis; d, its testicular component at higher magnification; e, cross section of right lobe of the testis of a primary male gonochorist, same magnification as in c.

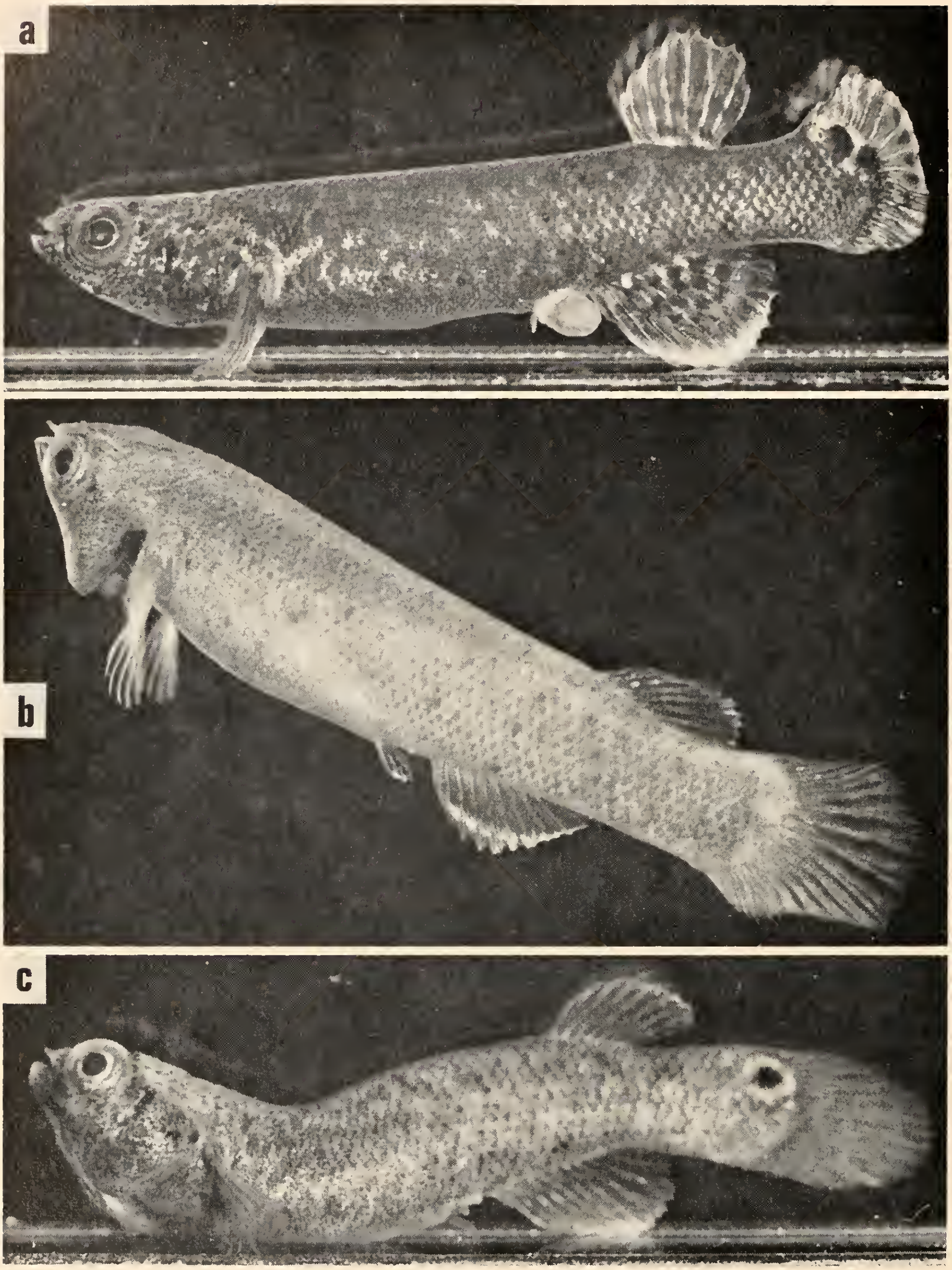

FIGURE 3. Structural-functional abnormalities of *Rivulus marmoratus* exposed to certain light-salinity-temperature combinations. Compare with Figure 1. a, *prolapsed oviduct;* confined to the dim-light, sea-water, high-temperature treatment; in 100% of the survivors; b, *pharyngeal hyperplasia;* confined to the dim-light, fresh-water, low-temperature treatments, whether begun at 18° C. and continued at 20° C., or at 20° C. throughout; in 100% of the

stat within 30 ± 1° C. Jars contained either filtered 100% sea water (salinity, 36‰) or bottled drinking water ("Blue Crystal"). Illumination was provided by four 40-watt Westinghouse Daylight fluorescent lamps suspended from above and controlled by a time switch giving a 14-hour photoperiod (0500h–1900h). Bright-lit jars received direct light of low daylight intensity (425–520 lux). Dim-lit jars were within a cube-shaped enclosure covered with black cloth on all but one side, either in a water bath or on shelves. They received indirect light of low intensity (2.55–21.00 lux), *i.e.*, mostly above the lower end of the intensity range (3.5–400.0 lux) of civil twilight (see Nielsen, 1961, 1963). Before hatching occurred, jars were examined once a day by flashlight; afterwards, although the hatchlings could feed by sight, each jar was taken out into the direct light for less than a minute each day to be checked and cleaned.

Results of Experimental Series One

Of the 150 eggs allotted to the eight treatments, 73 or 40.7% survived to sexual differentiation and functional maturity. Thirty-seven or 50.7% of these were hermaphrodites; 36 or 49.3% were males, an absolute number of males over seven times the total number encountered before the experiments were performed. All but one experimental male were from the low-temperature treatments (Table I). Figure 1 shows the incidence of males and hermaphrodites and the mortalities as percentages of the total number of eggs per treatment. In Table I, the data are given in actual numbers and also as percentages of survivors and non-survivors.

There is no indication that the incidence of males *versus* that of hermaphrodites was affected by the alternative salinities and light intensities in any of their four possible combinations, even though two light-salinity-temperature combinations resulted in far higher mortalities than the rest, *viz.*, either bright or dim light with sea water at 18° C. (see above). This is obvious from Figure 1, and warrants placing all high-temperature fish in one group and all low-temperature fish in another, as is done variously in Tables I–III. The correlation of male incidence with low-temperature rearing holds regardless of parentage or clone (Table II), or of embryonic stage at outset of treatment between Stages 1 and 13c (Table III).

There are indications, besides, that death was caused differently among the various light-salinity-temperature treatments, making it unlikely that the alternative temperatures *per se* caused alternative differential mortalities of hermaphrodites *versus* males. These indications are structural-functional abnormalities peculiar to certain light-salinity-temperature treatments. The names given them here, *prolapsed oviduct, pharyngeal hyperplasia,* and *kyphosis,* are intended to be no more than descriptive (Fig. 3). These abnormalities suggest that some deaths came from extreme expression of the abnormality or stress peculiar to the light-salinity-temperature treatment concerned.

Prolapsed oviduct was confined to the dim-light, sea-water, high-temperature treatment, and appeared in 100% of the survivors. It may be defined as oviposition into a non-patent, exserted oviduct. The oviduct protrudes from the genital

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survivors; c, *kyphosis;* of less than 100% incidence in the fish of two complementary treatments both with dim light, one with fresh water and high temperature, the other with sea water and low (18° C. changed to 20° C., and 20° C. throughout); often accompanied by non-buoyancy and thinness of body; commoner in the low-temperature treatments.

pore as a flaccid, blind sac, filled with expelled eggs that ultimately break down. The tip of one sac was snipped off, and subsequent ovipositions were successful. The abnormality finally corrected itself in the other fish, and each in the end produced viable hatchlings, as in all the other treatments. Prolapsed oviduct occurs infrequently among *R. marmoratus* routinely propagated, and occasionally is fatal.

Pharyngeal hyperplasia was confined to the dim-light, fresh-water, low-temperature treatments, whether begun at 18° C., and continued at 20° C. or at 20° C. throughout. It occurred in 100% of the survivors of these treatments, male or hermaphrodite. It shows externally as permanently raised opercula, gaping widely and exposing basibranchial swellings. One fish head was sectioned and found to have profuse thyroid tissue, some of it apparently usurping branchial cartilage, so that the condition may tentatively be diagnosed as thyroid hyperplasia, with the reservation that no control material was sectioned.

Kyphosis (Rasquin and Rosenbloom, 1954) shows best in roentgenogram or after clearing and staining with alizarin, but was intense enough here to show up externally, although mild cases may have eluded recognition. Unlike the other two abnormalities, it fell short of 100% occurrence and was often associated with non-buoyancy and thinness of body. Kyphosis occurred in fish of two complementary treatments both with dim light, one with fresh water and high temperature, the other with sea water and low temperature (18° C. changed to 20° C. and 20° C. throughout). It was more prevalent in the low-temperature treatments and accompanied several early deaths.

Apart from these plausible causes of death, a large portion of the fish died from failure to hatch (Table I), and some deaths were probably the delayed results of weakness caused by abnormally prolonged deferment of hatching.

Post-Treatment Observations

Eggs entered treatments August 9–November 15, 1961; treatments ended January 9–May 16, 1962. Post-treatment monitoring extended to June 1, 1965, when the last survivors were fixed for sectioning. Males and hermaphrodites died or were killed each year of the post-treatment observation period, 1962–1965. Males remained unchanged except for senile degeneration, but each year some hermaphrodites transformed to secondary male gonochorists (see Material and Methods). All fish were autopsied. Eight primary males, all of the secondary males, and all but one hermaphrodite were serially sectioned. Testes of secondary males are larger than those of primary males and apparently yield more spermatozoa.

Nearly 60% of the 37 original hermaphrodites had become secondary males by the time the last fish were killed at the end of May, 1965. Possibly some that died earlier and some killed at the end of the post-treatment observation period might also have changed over had they lived longer. Of the 16 fish dying as hermaphrodites, eight died natural deaths, three of them egg-bound; of those killed, five were egg-bound and probably doomed although otherwise healthy when killed, while the remaining three were healthy when killed at the end of May, 1965.

The original 37 hermaphrodites, including those later transforming into secondary males, were kept alive, isolated and under daily observation, for 262–1376 (average, 1041) days post-hatching; the original 36 males, for 327–1314 (average, 945) days. The days from first to last egg laid by hermaphrodites dying as such were 46–1117 (average, 694), by hermaphrodites later changing to secondary males, 107–1167 (average, 711). Secondary males acquire orange pigmentation like that of primary males, sometimes lose the caudal ocellus, which otherwise becomes obsolescent, but retain the basic hermaphrodite color pattern not shared with primary males, and are easily distinguished from primary males. The male attributes appear gradually, making their earliest recognition variable, so that the change from functional hermaphrodite to functional secondary male is best dated retrospectively, as the day on which the last egg was evacuated. Secondary males were kept alive beyond the day on which the last egg was laid (last day as hermaphrodite) for 48–918 (average, 346) days. No secondary male gonochorist reverted to its former (hermaphrodite) state. The serial sections provided detailed evidence of the transformation of ovotestes into testes. There was no histological evidence of change in the opposite direction.

The modalities of this sex inversion, from hermaphrodite to secondary male gonochorist, will be analyzed more closely in a separate report. Primary sex determination and differentiation are the concerns of the present report. These are distinct from the phenomenon of sex inversion, and with one exception this sex inversion took place after a life as a functional hermaphrodite as long as might be expected to be the life span in the wild of a fish species of this small size.

Experimental Series Two

Although the mortalities incurred in Experimental Series One (Fig. 1 and Table I) posed the formal dilemma of a differential mortality of hermaphrodites at low temperature and of males at high *versus* an experimental induction of males by low temperature and of hermaphrodites by high, nevertheless, the experiments gave strong presumptive evidence of an induction of males by low temperature. The dilemma concerned here usually presupposes a sex-determining mechanism producing about 50% females to 50% males under optimum conditions. This is clearly lacking or inoperative, because under routine laboratory conditions hermaphrodites preponderate over males, no females have been encountered at all, and only hermaphrodites have been encountered in the wild so far. On empirical grounds the expectation is therefore not males and hermaphrodites (or females) in equal numbers but rather a preponderance of hermaphrodites, an expectation not out of line with the mode of reproduction of this species or with the known genetic uniformity of the experimental fish.

The second series of experiments were undertaken to reconfirm the correlation of male incidence with low-temperature rearing, resolve the dilemma of a selective mortality of hermaphrodites *versus* induction of males at low temperature, and delimit somewhat, if existent, that segment of ontogeny within which low temperature can cause a deviation to the male phenotype. These objectives required fewer treatments with more individuals and lower mortalities than in the first series. The three treatments of 50 individuals each were essentially three different temperature regimes (Table IV, A–C). All 150 individuals received light of low daylight intensity. All were reared in fresh water until large enough (see above) to be fed brine shrimp, which were introduced in 40% sea water, after which the fish were gradually changed to 40% sea water, in which they spent the rest of their lives.

The individuals of Group A (controls) were reared throughout at a water temperature of 25 ± 1° C. Those of Group B were under the same conditions through hatching, but the hatchlings were reared for their first five months at 19.5 ± 0.5° C., after which they were maintained at 25 ± 1° C. Those of Group C were reared at 25 ± 1° C. until the eggs were at least in Stage 16 but not beyond Stage 22a (*cf.* Harrington, 1963); then they were reared through eclosion (hatching or cutting out of the chorion) and for five months post-eclosion at 19.5 ± 0.5° C., after which they were maintained at 25 ± 1° C. At the outset of the 19.5 ± 0.5° C. interval of their treatment, 28 of the Group-C individuals that survived to maturity were in Stage 16 (optic vesicles first visible as expansions of the forebrain), 8 in Stage 17 (optocoeles), 3 in Stage 19 (optic cup, lens, and neurocoele), 3 in Stage 20a (optic lobes, neuromeres, pectoral fin-bud anlagen), 3 in Stage 20b (heart pulses without blood circulation), and 1 in Stage 22a (circulation starting through dorsal aorta and vitelline vessels).

RESULTS OF EXPERIMENTAL SERIES TWO

The three objectives of this experimental series were achieved with decisive results (Table IV). The zero mortality and absence of males at 25 ± 1° C. (Group A) confirms the correlation of hermaphrodite incidence with high-temperature rearing while showing that the temperature need not be as high as 30° C., as in the first experimental series. This accords with the production of over 95% hermaphrodites to under 5% males when temperatures fluctuated more widely about 25° C., during routine rearing, suggesting that the low percentages of males hatched prior to these experiments resulted from temperatures temporarily below a threshold within 19–24° C. while these fish were traversing a critical segment of organogenesis thermolabile with regard to sex determination and differentiation. The correlation of male incidence with low-temperature rearing was established unequivocally (Group C).

The dilemma of a selective mortality of hermaphrodites *versus* induction of males at low temperature and *vice versa* at high, is resolved, because the mortalities incurred in Groups A–C were zero, and only 8% and 10%, respectively. There can be no doubt that low-temperature rearing caused a deviation to the male phenotype during a critical phase of ontogeny.

The possible extent of this thermolabile phenocritical period of sex determination and differentiation has been contracted at either end. Group B, with 92% survival and all hermaphrodites, not only reinforces the results of Group A, but shows that the thermolabile interval concerned does not extend beyond the end of Stage 31, the last stage before hatching. Group C showed that cold treatment need not begin before Stage 22a (blood circulation just established) to be effective in producing males. This phenocritical period may span a much shorter segment of ontogeny than its above-determined possible maximum extent. Only further experiments of similar design can define it more closely.

DISCUSSION

Uniqueness of the present experiments and results

Of previous experiments investigating possible influences of extrinsic factors on sex determination in fishes, two concerned that rather forced example of environmental influence, overripeness of eggs. In rainbow trout with a delayed fertilization of 21 days, Mršić (1923) reported 55% males, 33% females, and 12% interpreted by him as having ovaries transforming into testes, an interpretation put in doubt by later studies (see below, and Atz, 1964). With moderately delayed fertilizations, he reported small excesses of females. The controls deviated little from the 1:1 sex ratio. Mortalities were more than enough, however, to create the dilemma stated in the introduction to this report: 88% with the 21-day delay, 60–70% with moderate delays, 54% of the controls. Mršić dismissed the dilemma by discounting the mortality as having occurred too early in ontogeny to be the deciding factor, in an argument based on an histological interpretation (see above) of the course of gonad differentiation negated by later studies on rainbow trout (Padoa, 1939) and other salmonids (Ashby, 1952; Robertson, 1953). In brown trout from late-fertilized eggs Huxley (1923) found no significant departure from the 1:1 ratio.

Among broods of Siamese Fighting Fish, Eberhardt (1943) found the sex ratios extremely variable, but under optimum conditions approximating the 1:1 ratio. By crowding during rearing he obtained statistically significant excesses of males, and concluded that poor ("schlechte") space, food, and water conditions favored differentiation in the male direction, *i.e.*, opposite the genetic constitution. Nevertheless, a selective mortality of females cannot be ruled out, because he did not record the mortalities incurred in the experiments. Eberhardt rejected a selective elimination of females not on the basis of the experiments themselves but by inference from the results of rearing 25 other broods on scant food, so as to exaggerate the usual high mortality of the first two weeks of life. Only 4–47 fish survived from these broods of 100–400 hatchlings, and were well fed after the first two weeks. The survivors of 12 broods were 41–50% females; the other broods had somewhat lower percentages of females, but Eberhardt omitted details. The percentages of females in these underfed broods do not adequately support his contention that because in the experiments deaths did not exceed 1% after the first two weeks a selective mortality of females is ruled out. Furthermore, underfeeding and crowding cannot *a priori* be equated with regard to selective mortality, nor can either *a priori* be assumed without influence on sex determination.

A literature too large for more than summary treatment, reviewed in part by Dodd (1960) and Atz (1964), but much of it obsolete, concerns environmental influence on sex determination in the European eel. Grassi (1919) considered temperature, salinity, and nutrition to exert such influence. Forty years later, his student D'Ancona (1960; p. 67) was able to assert merely that his "own experiments suggest the possibility of a phenotypic sex deviation under the influence of experimental factors." Counter to an earlier report (D'Ancona, 1950) that sex ratios ascribed to environmental influences were attributable to a differential migration of the sexes, he named crowding and high temperature as "favoring differentiation toward the male sex," but the evidence is inconclusive and has since been put further in doubt (Sinha and Jones, 1966). It is unfortunate that a species so ill-suited for settling the question of environmental influence on sex determination in fishes became so closely linked with the question historically.

Each *Rivulus marmoratus* individual in the present experiments was reared *ab ovo* in its own container to preclude results attributable to crowding, which would be indecisive as to the proximate extrinsic causal factor. A freemartin-like effect cannot be dismissed as a possible result of crowding, for not only can sex steroids administered *per os* within a brief period of early ontogeny produce phenotypes of either sex in opposition to the genotypic sex (Yamamoto, 1953–1961), but there is evidence (Egami, 1954) for the uptake by fish in close confinement of estrogenic substances released by other fish (also *cf.* comments of Lindsey, 1962; p. 304). Among the fish crowded by Eberhardt (1943), for instance, rates of growth and sex differentiation varied so much that he resorted to removing the faster-growing ones when these could be sexed externally, each time reconcentrating the sexually indistinguishable ones remaining.

The induction of male gonochorists by incubating the eggs of *R. marmoratus* at low temperature detracts from the proposition that in eels high temperature favors sex differentiation in the male direction, by demonstrating an environmental effect on at least one species of fish the opposite of that on amphibians, in which it is well established (Witschi, 1929, 1957; Piquet, 1930; Uchida, 1937) that high temperature is male-inducing. *R. marmoratus* was chosen for its hermaphroditism and rare male incidence as possibly having a less homeostatic sex-determining mechanism than gonochoristic fishes and thus being a more likely species for testing for environmental influence on sex determination, but the results obtained with *R. marmoratus* raise expectations of analogous results with gonochoristic species of fish. Observations in harmony with our results, but directed to other ends and not excluding a selective mortality of the opposite sex, have in fact been made on two gonochoristic fishes of promise for such experimental testing. In exploring ways of rearing the cyprinodontid fish, *Epiplatys chaperi*, Van Doorn (1962) obtained a higher percentage of males at low temperatures. In experiments on meristic variation, Lindsey (1962) found that rearing conditions of high temperature and crowding produced higher percentages of female sticklebacks, *Gasterosteus aculeatus*.

The activity period of the sex-chromosome genes governing sex determination is equated by Atz (1964; p. 215) with the period of ontogeny in which it is possible with heterotypic hormones to reverse the sex of a gonochoristic fish, *e.g., Oryzias latipes* (Yamamoto, *loc. cit.*). Atz remarks that at present it is problematic whether a similar limited period could be ascribed to hermaphroditic species. Our results bear indirectly on this question. Sex reversal in the sense of transformation from one sexual phenotype (primary gonochorist) to the alternate one (secondary gonochorist) is not concerned here, so that in either hermaphrodite or gonochorist the interval by definition would not extend later than through the sexually indifferent and primary sex-differentiation stages. Both *O. latipes* and *R. marmoratus* hatch sexually undifferentiated and start eating at once as do other cyprinodontids. Making use of these traits by feeding sex steroids to *O. latipes* from the day *after hatching,* Yamamoto caused sex reversals in the sense of producing primary gonochorists of either sex contrary to genotypic sex. In the ontogeny of *R. marmoratus*, the interval within which low-temperature rearing produced males in opposition to the presumed hermaphrodite genotype (see above) begins after onset of blood circulation (possibly long after) but ends *before hatching.* It remains to be determined whether the thermolabile phenocritical period of sex determination in *R. marmoratus* is paralleled, overlapped, or succeeded by a hormonal lability in this respect. In the same context, although the post-hatching hormonal lability of *O. latipes* is not paralleled by a post-hatching thermal lability in *R. marmoratus,* the peroral administration of sex steroids by Yamamoto in excluding pre-hatching effects, leaves unknown whether the hormonal lability of *O. latipes* begins soon enough to parallel or overlap the pre-hatching thermal lability of *R. marmoratus*. In any case, the much shorter thermolabile interval of sex determination in *R. marmoratus* can be identified with the activity period of the sex-determining genes with as much reason as the interval of 8–10 weeks post-hatching during which Yamamoto fed *O. latipes* the steroids that caused sex reversals. This opens the possibility that teleostean sex determination entails a two-stage sex differentiation, the first stage with thermal lability, the second with hormonal lability.

Exclusion of alernative explanations

The male-inducing effect on *R. marmoratus* of incubation at low temperature emerges as a thermal effect apart from and undisturbed by the structural-functional abnormalities (prolapsed oviduct, pharyngeal hyperplasia, kyphosis) produced by certain (*vide supra*) specific light-temperature-salinity *combinations* of Experimental Series One. The same effect was achieved without these abnormalities, moreover, in Experimental Series Two, which avoided extremes of light intensity, temperature, and salinity, except for low temperature. These abnormalities were confined to dim-light treatments, and are attributable in part at least to hormonal derangements, which further indicates the independence and priority of the thermal effect on sex determination in *R. marmoratus*. Prolapsed oviduct results presumably from either precocious ovulation or abnormal persistence of non-patent oviduct, and in the European Minnow, *Phoxinus phoxinus*, for example, the oviduct becomes patent only within the spawning season, under endocrine control (Bullough, 1939). The pharyngeal hyperplasia and kyphosis in *R. marmoratus* kept at light intensities mostly within the range of civil twilight are reminiscent of the thyroid hyperplasia and kyphosis in the characin, *Astyanax mexicanus*, kept in total darkness, and ascribed to hormonal imbalance normally inhibited by light and involving but not confined to the pituitary-thyroid complex (Rasquin and Rosenbloom, 1954). The dim-light treatments of *R. marmoratus* began right after oviposition, at embryonic stages (Table III) not later than Stage 13c ($\frac{3}{4}$ blastoderm), but eggs of *A. mexicanus* spawned in the light failed to develop in the dark. Rasquin and Rosenbloom placed in darkness specimens kept in the light their first two months of life. Other causes of spinal curvature (Comfort, 1960, 1961) may also have been involved, because not all *A. mexicanus* kept in darkness showed kyphosis, and kyphosis was confined to *R. marmoratus* of only two dim-light treatments, in each of which it fell short of 100% occurrence.

Despite the evident primacy of thermal influence on sex determination and differentiation in *R. marmoratus*, it would constitute the fallacy of misplaced concreteness to conclude that males were produced by low temperature to the complete exclusion of influences from other extrinsic factors. The *principle of complementarity* as extended to biological phenomena (Meyer-Abich, 1956) is especially relevant to environmental influences on the ontogenetic differentiation of aquatic poikilotherms. To identify such influences requires polyfactorial analysis, with combinations of factors controlled as in the present experiments and in such as those of Kinne and Kinne (1962), who observe that not only can one environmental factor modify the physiological effect of another, but a single factor reaching sufficient intensity to modify the process under study may alter other environmental factors.

The obvious uncontrolled, dependent, extrinsic factor in these experiments on *R. marmoratus* is dissolved oxygen, each egg having been incubated in its own jar of stagnant water. Kinne and Kinne found stagnant (non-aerated) water to have 70 ± 10% the concentration of dissolved oxygen in aerated (100% air-saturated) water. From their nomograph (Kinne and Kinne, 1962, Fig. 2) can be obtained the approximate 100% air saturation (ml. O_2/L.) for each temperature-salinity combination of our experiments, except those with temperature changed from 18° to 20° C. Although the actual concentrations were less because the water was stagnant, the 100% air saturation values permit an arrangement of the experimental data in order of increasing oxygen concentration (Table VI). In contrast to the decisive thermal influence on sex determination in *R. marmoratus*, not only do these oxygen values fail to uncover evidence of an effect ascribable to oxygen concentration, but in a pilot experiment 10 eggs incubated at 25° C. in 100% air-saturated fresh water yielded 10 hermaphrodites.

TABLE V

Extraparental incubation periods of Rivulus marmoratus *with various combinations of light intensity, salinity, and temperature.* B, bright light; D, dim light; S, sea water; F, fresh water. Same eggs as in Tables I–III; hatched unaided (starred), light-triggered hatching (unencumbered numerals), cut from chorion (parentheses), started at 18° C. and changed to 20° C. (*italicized* numerals), the rest at 20° C. throughout

Numbers of days	Numbers of hatchlings												
	30° C.					18–20° C.							
	Hermaphrodites				Male	Hermaphrodites				Males			
	BS	DS	BF	DF	BF	BS	DS	BF	DF	BS	DS	BF	DF
12	1*												
14	3*	1*	2	2									
15–16	1*	4*		2									
18–19	1*		1	1									
23–24				2									
26–27			3		1								
28								_1_*					
30–31				1					1				1
35–36						2+1*		(1)	(1)	1		(1)1	
37–38							1			(1)	(2)1		
39–40							1	(1)1		1	2	1	(1)1
41–42										(1+_1_)	(1)	1	1+1*
43–44										(1)	_1_		
45–46						(1)				(1)	(3)	_2_	_2_
51–56												(_1_)	(_4_)
	14.5	15.0	21.0	19.4	27.0	39.0	38.5	35.3	33.5	40.7	41.0	42.1	44.0

Mean extraparental incubation periods in days.

Significance of the temperature effect per se

The production of male *R. marmoratus* by low-temperature incubation allows its examination in relation to corresponding rates of embryonic development. The developmental rates of *Cyprinodon macularius* exposed to a diversity of temperature-salinity-oxygen combinations were measured by Kinne and Kinne (1962) as numbers of days from fertilization to certain embryonic stages, especially hatching. These rates increased with increasing oxygen content, and decreased with increasing salinity, the latter effect mediated by changing coefficients of oxygen absorption and saturation in water. Both the retardation and the acceleration were increasingly accentuated by increase in temperature. For comparisons of developmental rates among *R. marmoratus* eggs exposed to different extrinsic factor combinations we must rely on the incomplete data (Table V) of Experimental Series One, because in Experimental Series Two exposure to low temperature was begun at a later and wider range of embryonic stages and the low-temperature embryos were cut from their chorions far in advance of the normal hatching stage (see above and Table IV, C). In Experimental Series One some eggs hatched unaided, others, with artificial stimulation; the embryos of the rest were cut out but only after some of the same age had hatched, unaided or aided (Table V). Intraparental (pre-treatment) incubations ranged at most from one to 24 hours (Table III and Harrington, 1963, Table I), treatment starting right after oviposition. The incubation periods of Table V might have diverged somewhat more had not many of them been ended arbitrarily, but most of the embryos cut out or from eggs stimulated to hatch would otherwise have perished unidentifiable as to ultimate sex type, as attested by mortalities (Table I) ascribable to extraparental incubations protracted by failure of the hatching mechanism.

TABLE VI

The results of Experimental Series One and Two arranged in order of increasing oxygen concentration at 100% air saturation. The actual concentrations were less, because the water was stagnant. See Discussion and Tables I and IV

Approximate temperature	30° C.	20° C.	30° C.	25° C.	20° C.	20° C.
ml. O_2/L. (100% air sat.)	4.3	5.3	5.6	6.0	6.6	6.6
Experimental Series	One	One	One	Two	One	Two
Total eggs	17	31	29	50	26	50
Percentage survival	64.7	67.7	51.7	100.0	53.8	90.0
Percentage male	0.0	48.0	3.4	0.0	30.8	72.0
Percentage hermaphrodite	64.7	19.7	48.3	100.0	23.0	18.0

The data of Table V, however imperfect, suffice to show a more delayed hatching at high temperature in stagnant fresh water than in stagnant sea water, which is paradoxical with reference to the eggs of *Cyprinodon macularius* (see above and Kinne and Kinne, 1962, Table X). Even if this perhaps resulted from impaired responsiveness of the hatching mechanism, it is no less interesting to find that the single, anomalous male produced at high temperature in our experiments had an extraparental incubation of 27 days in contrast to an average of 17 for the hermaphrodites otherwise produced at 30° C. The arbitrary curtailment of the incubations of many of the eggs at low temperature permits only the general comment that there are indications of a possible tendency toward longer incubations among the eggs later found to have yielded males and that the incubations at 20° C. were abnormally long for *R. marmoratus*. At the latitude of the wild-caught founder stock, which is at or near the northernmost extent of the geographic range of this chiefly tropical species, air-temperature daily minima between mid-April and mid-October form a plateau at 20° C., the daily means and maxima being much higher, of course. Most of the potential extent of the as yet undefined natural spawning season of *R. marmoratus* is excluded thereby from temperatures of sufficient duration low enough to produce males. Sooner or later, however, some males may be expected to be found in the wild at this latitude hatched from eggs incubated at lower temperatures toward the extremities of the spawning season or perhaps subjected to less obvious, alternative male-inducing conditions like the anomalous, lone male obtained at 30° C. (Tables I–III, V and VI).

The complex effects of temperature *per se* on morphological differentiation and the consequent impossibility of exactly equating developmental stages between embryos incubated at contrasting temperatures hardly needs stating. The imprecision of the classical embryonic "stage" was illustrated by Hayes (1949) with the comment added that hatching itself is not to be regarded as a stage, because it can occur so variably. Nevertheless, with cautionary reservations and for want of anything better, use must still be made of such "stages," sometimes even hatching, as was done by Kinne and Kinne (1962). Hatching as a stage is of normative importance here only in that experiment (Table IV, B) of Experimental Series Two in which low temperature treatment began with hatchlings from eggs incubated at our standard laboratory temperature (25 ± 1° C.). The mean extraparental incubation of the eggs of Table IV, B was 15.3 ± 3.7 days, the total incubation (extra- plus approximate intraparental) was 17.2 ± 3.7 days, and the feeding of each hatchling for one full day at 25 ± 1° C. gave a mean of 19.4 ± 3.8 days before transfer to low temperature. Accumulated laboratory records for 190 other eggs incubated at 25 ± 1° C. yielded a mean extraparental incubation of 17.3 ± 4.5 days and an approximate total incubation of 18.6 ± 4.4 days.

The very phenomenon under consideration, *viz.*, the production of males by extraparental incubation at low temperature, may itself be the result of an uncoupling of embryonic processes (*cf.* Hayes, 1949) by differential effects of low temperature on two or more constituent rates of development, so as to change the order of morphological events critical for sex differentiation. A paradigm for such an effect is the delay by low temperature of medullary development in amphibian gonads that feminizes males, at least temporarily (Uchida, 1937; Witschi, 1957). Although *medulla* as a topographic term has been declared inapplicable to teleostean gonads (D'Ancona, 1952), the bipotential gonocytes are sexualized as ovogonia and spermatogonia, respectively, in heterologous somatic territories within the ovotestes of several hermaphroditic fishes. Nor has uncertainty over the embryogenesis of the heterologous tissues deterred postulations of inductor substances in fishes analogous to the *corticin* and *medullarin* of Witschi, *viz.*, *gynogenine* and *androgenine* by D'Ancona (1949), *gynotermone* and *androtermone* by Yamamoto (1962).

Implications for the interpretation of intersexuality in fishes

Past studies of hermaphroditic fishes have been based at best on histological sections of gonads from economically feasible numbers of fish, sampling as wide a size range as collections provided. In most cases size was the sole criterion of relative age, an unreliable one for fishes, because there may be differential growth rates and mortalities between the sexes, including determinate *versus* indeterminate growth. Interpretations of otherwise adequate samples have been rendered inconclusive or incomplete by uncertainty over the relative ages of the fish coupled with the fact that the effects on growth rate of sex inversion and reversal are unknown. These difficulties are avoided with *R. marmoratus,* which is the first hermaphroditic fish species to have been kept in the laboratory throughout life. The results of the present experiments in conjunction with the daily observation of the fish of Experimental Series One throughout their lives throw light on aspects of fish intersexuality hitherto obscure, because the age and history of each fish were known exactly.

Before applying the results of the present study to these aspects of fish intersexuality, it is pertinent to reassess the extent to which the life span of *R. marmoratus* was encompassed by the experimental and post-experimental observations of Experimental Series One. Several tokens of senility (Comfort, 1960, 1961; Walford and Liu, 1965) appeared among these fish, *e.g.*, clouded cornea, emaciation, exophthalmos, humped back, raising of scales, renal concretions. They were kept alive as long as 1,376 days post-hatching; most of those killed were already *in extremis.* The life span of *R. marmoratus* seems to be of the order of that of another cyprinodont, the poeciliid *Lebistes reticulatus.* Under laboratory conditions, *Lebistes* has a limiting age of 2,000 days, 50% of age-dependent deaths occurring by the end of 800–900 days (Comfort, 1961). Survival both of *Rivulus* and of *Lebistes* to the more advanced ages reached in the laboratory (see Post-treatment Observations) is probably negligible in the wild.

Rivulus marmoratus is the only fish species known to exemplify the ultimate mode of synchronous hermaphroditism, normal self-fertilization. The only other synchronously hermaphroditic fishes known are the serranids, *Serranus cabrilla, S. hepatus, S. scriba* and *S. subligarius,* none of which are claimed to be naturally self-fertilizing (Clark, 1959; Reinboth, 1962; Atz, 1964). The life cycle of *S. subligarius* is incompletely known. The other three species mature and function first as males, the next year first functioning as synchronous hermaphrodites, but it is not known whether all start out as males or some start as hermaphrodites their first year (Reinboth, 1962). On the other hand, *R. marmoratus* is self-fertilizing from outset of functional maturity, even when this is precocious, at higher than usual temperatures. In a sense, therefore, neither these serranids nor *R. marmoratus* are obligate synchronous hermaphrodites throughout life, some or all of the serranid individuals serving as males their first year and some individuals of *R. marmoratus* ending their lives as functional secondary male gonochorists, at least under life-prolonging laboratory conditions. The distinction between synchronous and successional hermaphroditism in fishes thus seems to be one of degree. Of the two forms of successional hermaphroditism, protogyny and protandry, protogyny is the one toward which the synchronous hermaphroditism of *R. marmoratus* leans in tending toward transformation in the male direction, but protogyny is characterized by temporal succession to the functional male state from the functional female state and not from a fully functional synchronous hermaphroditism, primary and of long duration, as in *R. marmoratus*.

The induction of primary male gonochorists by low-temperature incubation, added to the spontaneous inversion of older hermaphrodites into secondary male gonochorists, are attributes of *R. marmoratus* recalling that primary and secondary males occur also among wrasses (Labridae), *e.g.*, *Coris julis* and *Thallasoma bifasciatum* (Reinboth, 1962). Although secondary male wrasses originate by sex reversal from a primary female condition instead of by sex inversion from synchronous hermaphrodites, perhaps all or some primary male wrasses arise from incubation at low temperature like the primary males of *R. marmoratus*, the no more likely alternative being a homogamety-heterogamety (*e.g.*, XX-XY) switch mechanism yielding protogynous hermaphrodites *versus* primary males, respectively, or *vice versa*. Wrasses have pelagic eggs of short incubation and spawning seasons (Breder and Rosen, 1966) such as to encourage a search for differential latitudinal occurrences of primary males correlated with differential thermal exposures of the drifting eggs. Control of male coloration, however, seems to differ between *Rivulus* and these wrasses. The inference of Zahl (1934), that the caudal ocellus of *Rivulus urophthalmus* is a sex-limited trait suppressed by testicular secretion is strengthened by its presence in all immature *R. marmoratus* also, and even more by its persistence in the hermaphrodites (Figs. 2 and 3), progressive extinction if these transform into secondary males, and usually complete disappearance in primary males (Figs. 2 and 3) at sexual maturity. The striking dichromatism between primary and secondary male wrasses seems to have a more complex hormonal control (Reinboth, 1962; comments of Atz, 1964).

Although *Rivulus marmoratus* exhibits a prolonged synchronous hermaphroditism, its sexuality is more like that of wrasses, *e.g.*, *Coris julis*, than of any other hermaphroditic fishes, with either synchronous or successional hermaphroditism. As far as they have been described, hermaphroditic fishes other than the wrasses and *R. marmoratus* have structurally bisexual gonads alone, although one or the other of the heterologous gonadal territories or dispersed centers may be in a state of persistent abortiveness (rudimentary hermaphroditism), prefunctional latency, or postfunctional involution. The wrasses and *R. marmoratus* also have gonads of bisexual structure. In the wrasses, these function first as ovaries, then, after a short transitional period, as testes, their possessors changing from functional females to functional secondary males. In *R. marmoratus,* they function from the first as ovotestes (Fig. 1, c and d), then after involution of the ovarian component and further evolution of the testicular, as testes alone, their possessors changing from self-fertilizing hermaphrodites for the greater part of their lives to functional secondary male gonochorists for the remainder. Unlike all other hermaphroditic fishes, however, these wrasses and *R. marmoratus* include, besides individuals with bisexual gonads, a minority of primary male gonochorists, with testes of unisexual structure (Fig. 2, e), in contrast to the bisexual structure of the testes of secondary male gonochorists.

The testes of primary males of *R. marmoratus*, and of *Coris julis* (Reinboth, 1962), are like those of the true gonochoristic species making up the majority of fishes, and can easily be told apart from testes of secondary male gonochorists, which retain the oviduct functional during the female or hermaphroditic phase of the bisexual gonad, and often ovarian residua besides. Testes of true gonochoristic fishes and of the primary males of *R. marmoratus* and *Coris julis* in being of unisexual structure, without oviducal or ovarian vestiges, differ also from the testes of protandrous fishes and of nominal gonochoristic fishes functionally gonochoristic throughout life but with incipient bisexual structure. Testes of the last two categories are so similar that Reinboth (1962, Fig. 27; reprinted as Fig. 2 of Atz, 1964) refers both of them to the same cross-section diagram (Fig. 27, c), which stands in marked contrast to that for the testes of primary males of *Coris julis* (Fig. 27, a). What is labeled *oviduct* in Reinboth's diagrams is of dual origin, the anterior paired lumina derived from the entovarial sulci and nonhomologous with the intratesticular sperm ducts according to Eggert (1933), the unpaired posterior duct arising differently.

In the case of the wrasses, the alternative of primary male *versus* protogynous hermaphrodite might possibly be decided by a genetic shift mechanism instead of by low-temperature incubation, but this is not the case with *R. marmoratus*. In the critical and decisive Experimental Series Two, not only were all individuals of the same clone but they were the end products of selfing through 9–11 uniparental laboratory generations. Since then, further evidence (unpublished) for the homozygosity of these fish has been accumulating from hybridization-*cum*-grafting experiments. All of these lines of evidence of the overwhelming prevalence of hermaphrodites point to the likelihood that the fish of this clone are not only homozygous, but, inferentially, homogametic as well, both hermaphrodites and experimentally produced primary male gonochorists. The fact that both natural and experimentally induced departures from the modal phenotypic expression of the hermaphrodite genotype of *R. marmoratus* are in the male direction, makes it intriguing to find that among amphibians steroid hormones seem capable of causing sex reversal only in the homogametic sex, *i.e.*, to the phenotype of the heterogametic sex (Witschi, 1957). In the fish, *Oryzias latipes*, however, fully functional sex reversals in either direction are produced with sex steroids (Yamamoto, 1953–1961). In *R. marmoratus*, the hermaphroditic constitution is manifestly the epistatic one and the male constitution, the hypostatic. While males result from low-temperature incubation or from sex inversion late in life, females are non-existent. In pilot experiments in which eggs were incubated at a descending series of constant temperatures, 31.2° C. was the highest at which eggs survived, the 10 eggs concerned yielding 10 hermaphrodites.

Breder and Rosen (1966) construe the hermaphroditism of *R. marmoratus* as a mechanism evolved to compensate for the vicissitudes of the unstable coastal mainland and oceanic island environments, allowing reproduction in spite of severe population depletions by violent coastal storms. By the same tokens, if *R. marmoratus* proves to be hermaphroditic throughout its range, which arches island-to-island across the Caribbean from off the Venezuelan coast onto the Florida peninsula, it is easy to see how island-hopping hermaphrodite castaways might found new colonies more readily than males and females of gonochoristic species, with little chance of meeting. Haldane (1957) argues similarly for the colonization of new rivers by flood-transported hermaphroditic or parthenogenetic fish, while noting that if the selfed immigrant ancestor were homogametic the progeny would be more likely to die out, because of the absence of males. Whatever the long-range prospects of survival for *R. marmoratus*, the graft tests for histocompatibility between wild-caught progenitors and their earliest laboratory descendants make it inescapable that selfing had gone on in the wild for some time. In the long run, perhaps enough males (primary or secondary gonochorists) are produced in the wild to contribute genetic information occasionally from their clone to eggs of another clone, by mating with a hermaphrodite with ovotestes out of phase so as to emit unfertilized eggs. Hermaphrodites do occasionally emit a spate of unfertilized eggs (Harrington, 1963) and we have had apparent success in fertilizing a few of these with sperm from a male of different clone. Attempts to mate males with hermaphrodites have resulted in sexual coaction, but the hermaphrodites, whether previously isolated or not, have emitted eggs in stages of development so advanced as to indicate self-fertilization long before the pairing. In sum, amphimixis between eggs from hermaphrodites of one clone and sperm from primary or secondary male gonochorists of a different clone is in the realm of possibility, but the evidence so far is that it has occurred rarely if at all in the local populations from whence our stock was derived.

The writer thanks E. S. Harrington and L. A. Webber for their faithful technical assistance, Drs. C. M. Breder, Jr., K. D. Kallman, and M. W. Provost for reading the manuscript, Drs. J. B. Leonard and J. Packer for microphotographs, Mr. J. O'Neal for macrophotographs, and Mr. W. Janse for executing Figure 1. This is Contribution No. 167 of the Entomological Research Center, Florida State Board of Health, supported by PHS grant #ES 00161 from the Office of Resource Development, BSS (EH), and by PHS grant #CC 00274 from the Communicable Disease Center, Atlanta, Georgia.

SUMMARY

1. *Rivulus marmoratus* is the only known hermaphroditic fish species naturally self-fertilizing. Tissue grafts between wild-caught fish and their uniparental laboratory descendants give the *autograft reaction*, indicating propagation by selfing in the wild also. Only hermaphrodites have been found in the wild locally, although selfing through more than 10 uniparental laboratory generations yielded a few primary male gonochorists, under 5% in contrast to over 95% that were hermaphrodites. Females seem to be non-existent.

2. Two series of experiments were undertaken to identify a possible environmental factor able to cause a deviation to the male phenotype during sex differentiation, on the working hypothesis that low male incidence in clones composed otherwise of hermaphrodites indicated a lability in the sex-determining mechanism through which the genotype normally produces the hermaphrodite phenotype.

3. Individuals of two clones, each in its own jar throughout life, were exposed to the eight combinations of bright or dim light, sea water or fresh water, high or low temperature (Experimental Series One). Exposure was from not later than the ¾ blastoderm stage until sexual maturity at high temperature or five months post-hatching at low.

4. Over seven times the number of males previously encountered were obtained, all but one from low-temperature treatments. Male production was correlated with

low-temperature rearing despite alternative light intensities and salinities and structural-functional abnormalities (*prolapsed oviduct, pharyngeal hyperplasia, kyphosis*) peculiar to different dim-light, salinity-temperature combinations, and partly attributable to hormonal derangements. Mortalities were high enough to present the formal dilemma of a differential male induction *versus* hermaphrodite mortality at low temperature and *vice versa* at high, but this dilemma was resolved by Experimental Series Two.

5. The Experimental Series One fish were monitored daily up to 1,376 days post-hatching, by which time almost 60% of the hermaphrodites had changed to functional secondary male gonochorists, the rest dying or killed as hermaphrodites, some each year. Primary males remain unchanged except for senile degeneration. Secondary males arise mostly late in laboratory-prolonged life, by involution of the ovarian component of the ovotestes with further evolution of the testicular component, the caudal ocellus fading or vanishing as they become orange like the primary males.

6. In Experimental Series Two, mortalities were low and the structural-functional abnormalities were absent. All individuals were kept at the same intermediate salinity and light intensity: Group A, at moderate temperature throughout to maturity; Group B, at the same temperature through hatching, at low temperature the first five months post-hatching, thereafter at the moderate temperature; Group C, at the moderate temperature up to stages from optic vesicle formation to outset of blood circulation, then at low temperature through eclosion and for five months post-eclosion. Group-C embryos being cut from their chorions to minimize deaths from hatching failure.

7. The Group-A eggs yielded 100% hermaphrodites, the Group-B eggs, 92% hermaphrodites and 8% deaths, the Group-C eggs, 72% males, 18% hermaphrodites, and 10% deaths. Exposure to low temperature from as late as outset of blood circulation produced males.

8. The uniqueness of the present experiments and results, exclusion of alternative explanations, significance of the temperature effect *per se,* and the implications of these findings for the interpretation of intersexuality in fishes are discussed at length.

LITERATURE CITED

Ashby, K. R., 1952. Sviluppo del sistema riproduttivo di *Salmo trutta* L., in condizioni normali e sotto l'influenza di ormoni steroidi. *Riv. Biol.*, n. s. **44**: 3–18.

Atz, J. W., 1964. Intersexuality in fishes. *In*: Intersexuality in Vertebrates Including Man (ed. by C. N. Armstrong and A. J. Marshall), Academic Press, London and New York.

Breder, C. M., Jr., and D. E. Rosen, 1966. Modes of Reproduction in Fishes. The American Museum of Natural History, Natural History Press, Garden City, New York.

Bullough, W. S., 1939. A study of the reproductive cycle of the minnow in relation to the environment. *Proc. Zool. Soc. London, Ser. A,* **109**: 79–102.

Clark, E., 1959. Functional hermaphroditism and self-fertilization in a serranid fish. *Science,* **129**: (3343): 215–216.

Comfort, A., 1960. The effect of age on growth resumption in fish (*Lebistes*) checked by food restriction. *Gerontologia,* **4**: 177–186.

Comfort, A., 1961. The longevity and mortality of a fish (*Lebistes reticulatus* Peters) in captivity. *Gerontologia,* **5**: 209–222.

D'Ancona, U., 1949. Ermafroditismo ed intersessualità nei Teleostei. *Experientia,* **5**: 381–389.

D'Ancona, U., 1950. Détermination et différenciation du sexe chez les poissons. *Arch. Anat. Micros. et Morph. Exp.*, **39**: 274–292.

D'Ancona, U., 1952. Territorial sexualization in the gonads of teleosteans. *Anat. Rec.*, **114**: 666–667.

D'Ancona, U., 1960. The life-history of the Atlantic eel. *In*: Hormones in Fish (ed. by I. Chester Jones), *Symposia Zool. Soc. London*, No. 1.

Dodd, J. M., 1960. Genetic and environmental aspects of sex determination in cold-blooded vertebrates. *Mem. Soc. Endocrinol., No.* **7**: 17–44.

Eberhardt, K., 1943. Geschlechtsbestimmung und -differenzierung bei *Betta splendens* Regan. *Zeitschr. für indukt. Abstamm. und Vererbungslehre*, **81**: 363–373.

Egami, N., 1954. Influence of temperature on the appearance of male characters in females of the fish, *Oryzias latipes*, following treatment with methlydihydrosterone. *J. Fac. Sci., Univ. Tokyo*, Sec. IV, **7** (2): 281–298.

Eggert, B., 1933. Die Intersexualität bei Knochenfischen. *Zeitschr. wiss. Zool.*, **144**: 402–420.

Garman, S., 1895. The cyprinodonts. *Mem. Mus. Comp. Zool.*, **19** (1): 1–179.

Gordon, M., 1957. Physiological genetics of fishes. *In*: The Physiology of Fishes (ed. by M. E. Brown), Vol. 2, Academic Press, New York.

Grassi, B., 1919. Nuove ricerche sulla storia naturale dell'Anguilla. *Mem. R. Com. Talass. Ital.*, **67**: 1–141.

Haldane, J. B. S., 1957. Sex determination in Metazoa. *Proc. Zool. Soc. Calcutta*, Mookerjee Memorial Volume **1957**: 13–28.

Harrington, R. W., Jr., 1959. Delayed hatching in stranded eggs of marsh killifish, *Fundulus confluentus*. *Ecology*, **40** (3): 430–437.

Harrington, R. W., Jr., 1961. Oviparous hermaphroditic fish with internal self-fertilization. *Science*, **134** (3492): 1749–1750.

Harrington, R. W., Jr., 1963. Twenty-four-hour rhythms of internal self-fertilization and of oviposition by hermaphrodites of *Rivulus marmoratus*. *Physiol. Zoöl.*, **36** (4): 325–341.

Harrington, R. W., Jr., and L. R. Rivas, 1958. The discovery in Florida of the cyprinodont fish, *Rivulus marmoratus*, with a redescription and ecological notes. *Copeia*, **1958** (2): 125–130.

Hayes, F. R., 1949. The growth, general chemistry, and temperature relations of salmonid eggs. *Quart. Rev. Biol.*, **24**: 281–308.

Hoedeman, J. J., 1958. Rivulid fishes of the Antilles. *Studia Fauna Curaçao*, **8**: 112–126.

Huxley, J. S., 1923. Late fertilization and sex-ratio in trout. *Science*, **58** (1502): 291–292.

Kallman, K. D., and R. W. Harrington, Jr., 1964. Evidence for the existence of homozygous clones in the self-fertilizing hermaphroditic teleost *Rivulus marmoratus* (Poey). *Biol. Bull.*, **126**: 101–114.

Kinne, O., and E. M. Kinne, 1962. Rates of development of embryos of a cyprinodont fish exposed to different temperature-salinity-oxygen combinations. *Canad. J. Zool.*, **40**: 231–253.

Lindsey, C. C., 1962. Experimental study of meristic variation in a population of threespine sticklebacks, *Gasterosteus aculeatus*. *Canad. J. Zool.*, **40**: 271–312.

Meyer-Abich, A., 1956. The principle of complementarity in biology. *Acta Biotheoretica, Ser. A*, **11**: 57–74.

Mršić, W., 1923. Die Spätbefruchtung und deren Einfluss auf Entwicklung und Geschlechtsbildung, experimentell nachgeprüft an der Regenbogenforelle. *Arch. f. Entw.*, **98**: 129–209.

Nielsen, E. T., 1961. Twilight and the "crep" unit. *Nature*, **190** (4779): 878–879.

Nielsen, E. T., 1963. Illumination at twilight. *Oikos*, **14** (1): 9–21.

Nogusa, S., 1960. A comparative study of the chromosomes in fishes with particular consideration on taxonomy and evolution. *Mem. Hyogo Univ. Agric.* (Biol.), **3** (1): 1–60.

Padoa, E., 1939. Observations ultérieures sur la différenciation du sexe, normale et modifiée par l'administration d'hormone folliculaire, chez la Truite iridée (*Salmo irideus*). *Bio-Morphosis*, **1**: 337–354.

Pflüger, E., 1882. Versüche der Befruchtung überreifer Eier. *Pflüger's Archiv für gesamte Physiol. des Menschen und der Tiere*, **29**: 76–77.

Piquet, J., 1930. Détermination du sexe chez les batraciens en fonction de la température. *Rev. Suisse Zool.*, **37**: 173–281.

Rasquin, P., and L. Rosenbloom, 1954. Endocrine imbalance and tissue hyperplasia in teleosts maintained in darkness. *Bull. Amer. Mus. Nat. Hist.*, **104** (4): 365–425.

Reinboth, R., 1962. Morphologische und functionelle Zweigeschlechtlichkeit bei marinen Teleostieren (Serranidae, Sparidae, Centracanthidae, Labridae). *Zool. Jahrb. Abt. für Zool. und Physiol. der Tiere*, **69**: 405–480.

Rivas, L. R., 1945. The discovery and redescription of the types of *Rivulus marmoratus* Poey, a cyprinodont fish from Cuba. *J. Wash. Acad. Sci.*, **35** (3): 95–97.

Robertson, J. G., 1953. Sex differentiation in the Pacific salmon *Oncorhynchus keta* (Walbaum). *Canad. J. Zool.*, **31** (2): 73–79.

Rothschild, Lord, 1958. Fertilization in fish and lampreys. *Biol Rev.*, **33**: 372–392.

Sinha, V. R. P., and J. W. Jones, 1966. On the sex and distribution of the freshwater eel (*Anguilla anguilla*). *J. Zool., Lond.*, **150** (3): 371–385.

Sinnott, E. W., and L. C. Dunn, 1939. Principles of Genetics. 3rd ed., McGraw-Hill Book Co., New York and London.

Spurway, H., 1957. Hermaphroditism with self-fertilization and the monthly extrusion of unfertilized eggs in the viviparous fish *Lebistes reticulatus*. *Nature*, **180** (4597): 1248–1251.

Uchida, T., 1937. Studies on the sexuality of Amphibia. III. Sex-transformation in *Hynobius retardatus* by the function of high temperature. *J. Fac. Sci. Hokkaido Imper. Univ., Ser. VI*, **6**: 59–70.

Van Doorn, W. A., 1962. Geschlachtsbeinvloeding door temperatur. *Het Aquarium*, **32** (9): 208–209.

Walford, R. L., and R. K. Liu, 1965. Husbandry, life span, and growth rate of the annual fish, *Cynolebias adloffi* E. Ahl. *Exp. Geront.*, **1**: 161–171.

White, M. J. D., 1954. Animal Cytology and Evolution. 2nd ed., Cambridge University Press, Cambridge, England.

Witschi, E., 1929. Studies on sex differentiation and sex determination in amphibians. II. Sex reversal in female tadpoles of *R. sylvatica* following the application of high temperature. *J. Exp. Zool.*, **52**: 267–291.

Witschi, E., 1957. The inductor theory of sex differentiation. *J. Fac. Sci. Hokkaido Univ., Ser. VI*, **13** (Professor T. Uchida Jubilee Volume): 428–439.

Yamamoto, T., 1953. Artificially induced sex-reversal in genotypic males of the Medaka (*Oryzias latipes*). *J. Exp. Zool.*, **123**: 571–594.

Yamamoto, T., 1958. Artificial induction of functional sex-reversal in genotypic females of the Medaka (*Oryzias latipes*). *J. Exp. Zool.*, **137**: 227–260.

Yamamoto, T., 1959. The effects of estrone dosage level upon the percentage of sex-reversals in genotypic male (XY) of the Medaka (*Oryzias latipes*). *J. Exp. Zool.*, **141**: 133–154.

Yamamoto, T., 1961. Progenies of sex-reversal females mated with sex-reversal males in the Medaka, *Oryzias latipes*. *J. Exp. Zool.*, **146**: 163–179.

Yamamoto, T., 1962. Hormonic factors affecting gonadal sex differentiation in fish. *Gen. Comp. Endocrin., Suppl.*, **1**: 341–345.

Zahl, P. A., 1934. The hormonic inhibition of a somatic character in the cyprinodont fish, *Rivulus urophthalmus*. *Amer. Nat.*, **68**: 475–477.

RETINOMOTOR RHYTHMS IN THE GOLDFISH, CARASSIUS AURATUS[1]

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We had preliminary evidence from histological preparations that the retina of the goldfish, *Carassius auratus*, does show a persistent retino-motor rhythm in prolonged darkness, and we felt that the conclusions of Wigger (1941) stood in need of verification. Wigger (1941) placed the fish in darkness at 1800 hr. and sampled two fish at 2-hour intervals during the first day and at 1200 hr. and 2000 hr. the second day. According to his graph, the cones elongated smoothly to their positions of maximal dark-adaptation by 2200 hr. the first night, contracted sharply to a new position by 2400 hr. and remained there until 0400 hr. Then they contracted sharply to a location intermediate between the light- and dark-adapted positions by 0600 hr. and remained approximately in that position to the end of the experiment. Wigger's results demonstrated that the rhythmic migration of cones in the goldfish retina did not persist in constant darkness after the first 12 hours. He did not distinguish between types of cones.

It is well known that within a species the distances of migration of visual cells may vary considerably as do the thicknesses of the visual cell layers (Ali, 1963; Engström and Rosstrop, 1963) and that single and double cones migrate different distances (Walls, 1942; Nicol, 1965). In repeating these experiments on the goldfish, we followed the separate courses of migration of single and double cones.

Materials and Methods

The experiments involved 6 groups of goldfish (mean total length 6.6 ± 0.3 cm.) obtained from Nolt's Ponds, Silver Springs, Pennsylvania.

Group A: 82 fish, conditioned to the natural diel cycle, were placed in the darkroom at sunset, 2036 hr., on June 23, 1965. We fixed samples of 4 fish at 1-hour intervals between 2100 hr. and 0500 hr., and then at sunrise, 0534 hr. Thereafter, we fixed samples of 4 fish at 0800 hr., 1100 hr., 1300 hr., 1500 hr., 1800 hr., and 2036 hr.

Group B: 21 fish, conditioned as in group A, were placed in the darkroom at sunset, July 5, 1965. We fixed three fish at 1300 hr. for 7 consecutive days.

Group C: 12 fish were conditioned for 45 days on an artificial cycle, 12 hr. 20 min. light and 11 hr. 40 min. dark. The light was turned off permanently at 1910 hr. (sunset) on November 19, 1965, and for three consecutive days we fixed two fish at 2400 hr. and 1200 hr.

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[1] Supported by funds from the National Science Foundation (G-19256).

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Group D: 8 fish, conditioned as in Group A, were placed in the darkroom at sunset, June 13, 1966. We fixed two fish at 0100 hr. and 1300 hr. of the two consecutive days.

Group E: 18 fish, conditioned as in Group A, were placed in the darkroom at sunset on June 27, 1966. We fixed two fish at 1-hour intervals between 2100 hr. and 0500 hr.

Group F: 4 fish, conditioned as in group A, were placed in the darkroom at sunset on July 13, 1966. We fixed two fish at 0300 hr. and 0400 hr.

Light-adapted fish were fixed at 1200 hr. in July and November, 1965.

The fish were maintained in 77.5-liter aquaria equipped with filters and aerators. They were fed daily before noon though not at a regular hour. They were not fed during the experiments. The temperature was 25° ± 1° C.

The fish were fixed in Bouin's fluid in darkness (exclude light-adapted fish) and remained in the fixative at least two hours before the corneas and lenses were removed from the right eyes. The eyes were then dehydrated in an ethyl alcohol series, cleared in xylene, and embedded in paraffin with 1% beeswax.

In group C, radial and serial tangential sections were cut from a sector of retina 2 mm. square, located 1 mm. ventral to the optic nerve. In all other groups, sections were taken from the entire eye through the plane of the optic nerve on a dorso-ventral axis. All sections were cut at 5 or 10 μ and stained with Harris's haematoxylin and eosin. In order to expose the cones for accurate identification and measurement, some sections were bleached with the potassium permanganate-sodium bisulfite method, and stained with 3% ferric chloride and eosin.

The interpretation of the behavior of the retina was based upon the following measurements from each eye:

Group A: 5 measurements of the thickness of the visual cell layer, the location of the single cones, and the location of both the long and short segments of the double cones, all in a region of the retina 1.2 mm. ventral to the optic nerve. The visual cell layer was defined as the distance between the ELM (external limiting membrane) and the lamina basalis. The location of the cones was represented by the distance from the ELM to the distal end of the cone ellipsoid. All measurements were made with an ocular micrometer.

Group B: All measurements made as in group A.

Group C: Radial sections measured as in group A. On the serial tangential sections, using a Whipple-Hauser ocular micrometer, we counted the numbers of single and double cones in an area 670 μ square in each section beginning at the ELM and progressing to the lamina basalis. The distance between elements in successive sections was represented by the thickness of the sections, 5 μ.

Groups D, E, and F: The measurements were made as in group A except that 10 measurements were made rather than 5.

For final comparisons the measurements were converted to RP values (location of visual cell/thickness of visual cell layer × 100) described by Engström and Rosstrop (1963).

RESULTS

All graphs of double cones from radial sections represent the measurements on the long segment which had a mean length of about 3 μ greater than the short seg-

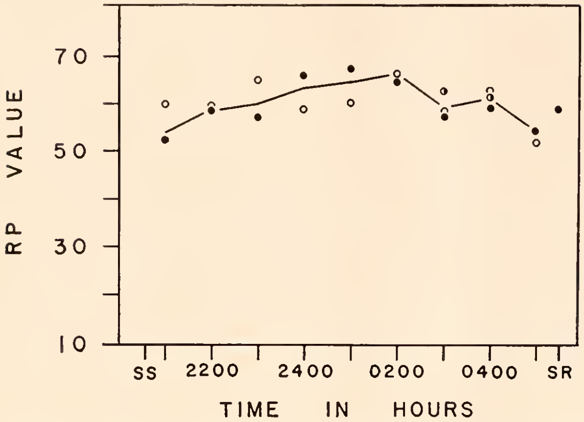

FIGURE 1. Pattern of migration of double cones during first night of darkness. ● Group A, ○ group E, and ◑ group F. SS = sunset, SR = expected sunrise, — = mean values.

ments. There was no change in the difference between the lengths of short and long segments for eyes fixed at different times. The patterns of photomechanical changes during the course of the first night are shown for double cones in Figure 1, and for single cones in Figure 2. Each graph is a composite of results from experimental groups A, E, and F. The points on the graph within a group are not based upon equal numbers of fish because some of the eyes did not produce useful histological sections. In Figure 1, the points for group A at 0100 hr., 0200 hr., and 0400 hr. represent three fish; all other points represent 4 fish. The point for group E at 2100 hr. represents one fish and all other points represent two fish. Each point for group F represents two fish. In Figure 2, the points for group A at 0100 hr. and 0400 hr. represent three fish; 0200 hr., two fish; all other points 4 fish. In group E, the point at 2100 hr. represents one fish, and all other points represent two fish. Each point for group F represents two fish.

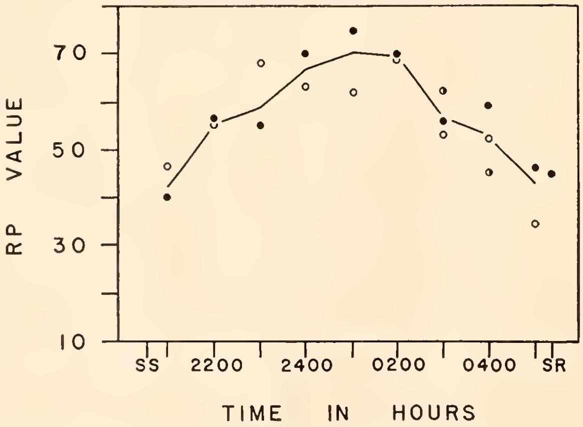

FIGURE 2. Pattern of migration of single cones during first night of darkness. ● Group A, ○ group E, and ◑ group F. SS = sunset, SR = expected sunrise, — = mean values.

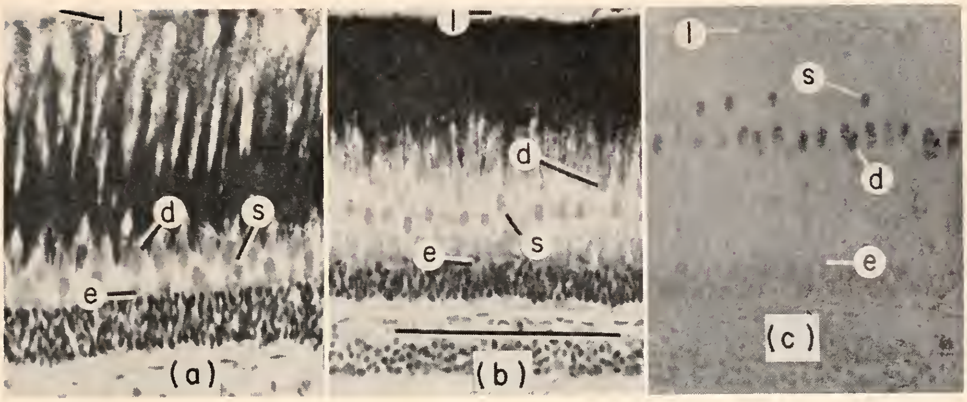

FIGURE 3. Photomicrographs of radial sections showing retinas in different states of adaptation. (a) Light-adapted, 10 μ, H&E. (b) Noon dark-adapted after 17 hours in darkness, 10 μ, H&E. (c) Midnight dark-adapted after 5 hours in darkness, pigment bleached; 5 μ, ferric chloride and eosin. The bar in (b) represents 100 μ. e = external limiting membrane, l = lamina basalis, s = single cones, and d = double cones. 450 ×.

The cones migrated through a cycle of positions during the course of the night, but in each group of fish they followed an irregular and unique course with maximal extensions occurring at different times in the night. Between 2100 hr. and 0200 hr., the corresponding points of groups A and E almost always deviated in opposite directions. The plot of the mean within that interval was relatively smooth. Between 0200 hr. and 0500 hr., the fluctuations in groups A and E followed parallel courses and both exhibited a prominent positive shift in the slope

TABLE I

Mean RP values of double cones during the first 24 hours in darkness, based upon radial sections. Sunset was at 2036 hr. and the expected sunrise was 0534 hr. Plus and minus values represent one standard deviation. The p values are based upon Student's t-test between successive time periods

Groups	Time	Number of fish	RP value	*p*
A, E, F	2100	5	53.6 ± 5.1	
	2200	6	59.2 ± 2.5	> .05
	2300	6	59.5 ± 4.8	n. s.
	2400	6	63.5 ± 5.8	n. s.
	0100	5	64.4 ± 4.1	n. s.
	0200	5	65.6 ± 3.4	n. s.
	0300	8	59.1 ± 6.9	n. s.
	0400	7	61.1 ± 4.4	n. s.
	0500	6	54.2 ± 2.9	> .01
A only	0534	4	59.3 ± 7.3	n. s.
	0800	3	45.1 ± 1.1	> .05
	1100	3	49.0 ± 3.6	n. s.
	1300	4	50.2 ± 3.9	n. s.
	1500	4	53.8 ± 6.4	n. s.
	1800	4	53.7 ± 8.4	n. s.
	2037	4	50.0 ± 3.8	n. s.

TABLE II

Mean RP values of single cones during the first 24 hours in darkness, based upon radial sections. Sunset was at 2036 hr. and the expected sunrise was 0534 hr. Plus and minus values represent one standard deviation. The p values are based upon Student's t-test between successive time periods

Groups	Time	Number of fish	RP value	*p*
A, E, F	2100	5	41.2 ± 8.0	
	2200	6	57.6 ± 6.8	> .01
	2300	6	59.3 ± 7.3	n. s.
	2400	6	67.8 ± 8.1	> .05
	0100	5	69.9 ± 7.8	n. s.
	0200	4	69.8 ± 3.0	n. s.
	0300	8	57.0 ± 6.7	> .01
	0400	7	53.2 ± 6.9	n. s.
	0500	6	42.8 ± 7.3	> .05
A only	0534	4	46.4 ± 13.0	n. s.
	0800	3	30.8 ± 3.7	n. s.
	1100	3	38.6 ± 0.7	> .05
	1300	4	31.3 ± 7.8	n. s.
	1500	4	32.6 ± 3.9	n. s.
	1800	4	30.7 ± 4.3	n. s.
	2037	4	30.3 ± 4.6	n. s.

of the curve between 0300 hr. and 0400 hr. The slope of the curve in that interval for group F was negative.

The single cones showed a greater photomechanical shift than the double cones. The photomicrographs in Figure 3 show that the distal margins of the ellipsoids of single cones were nearer the ELM in the 1300 hr. retina and farther from the ELM in the 0100 hr. retina than the double cones. The relative fluctuations between points in the graphs of single and double cones were about equal (Figs. 1 and 2), but the single and double cones may have shown some independent behavior.

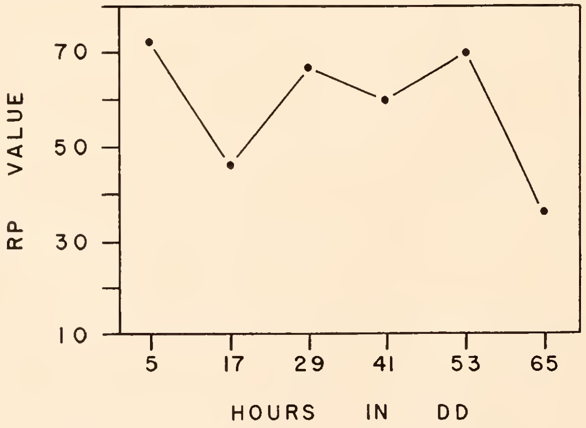

FIGURE 4. Rhythmic shift of positions of double cones based upon serial tangential sections. Group C.

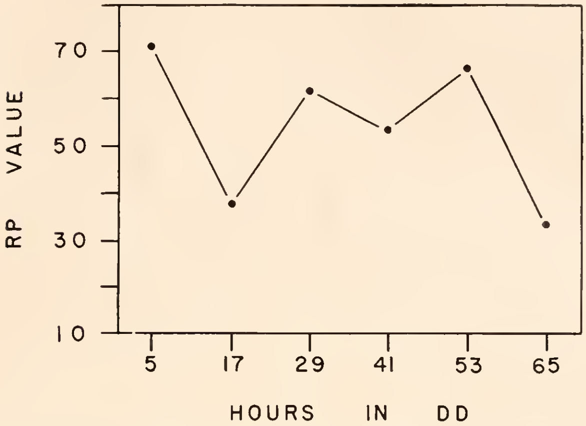

FIGURE 5. Rhythmic shift of positions of single cones based upon serial tangential sections. Group C.

Note in Figure 1 that only three of the points for group A lie above those of group E, while in Figure 2, 7 of the points for group A lie above those of group E.

Tables I and II summarize the combined statistical data for groups A, E and F. As shown by the probability values derived from Student's t-test, there were significant differences between the adjacent points from 2100 hr. to 2200 hr., 2300 hr. to 2400 hr., 0200 hr. to 0300 hr., and 0400 hr. to 0500 hr. The significant differences practically alternated with insignificant differences. The data for group A show that both single and double cones continued to contract beyond the time of expected sunrise. The RP values reached a minimum at 0800 hr. and fluctuated around that value through the remainder of the day.

Both single and double cones showed a persistent photomechanical rhythm in constant darkness. This was first demonstrated by group C (Figs. 4 and 5) in which the results were based upon serial tangential sections. Note that the

TABLE III

Mean RP values of double cones through 65 hours in darkness, based upon serial tangential sections. The eye at 0 hr. was light-adapted. Sections progress from the ELM toward the lamina basalis. Plus and minus values represent one standard deviation

Hours in DD	Fish number	Serial section sequence																			RP value
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	
0	1	0	34	50	21																20.9 ± 5
5	2	0	0	0	0	0	0	0	0	0	2	17	29	18							82.5 ± 6
5	3	0	0	0	0	0	0	13	25	35	26										61.3 ± 7
17	4	0	0	0	0	0	0	3	5	2	11	19	17	8							53.4 ± 8
17	5	0	0	0	4	7	10	12	17	20	14										38.0 ± 9
29	6	0	0	0	0	0	0	0	0	0	1	1	13	29	30	19					70.4 ± 6
29	7	0	0	0	0	0	0	0	0	0	0	1	5	17	38	29	21	3			66.2 ± 17
41	8	0	0	1	1	0	0	7	17	18	20	16	5								54.4 ± 10
41	9	0	0	0	0	0	2	0	2	1	0	8	19	17	17	17	3	4			64.0 ± 10
53	10	0	0	0	0	0	0	0	0	0	0	0	0	6	12	26	46	37	21	1	72.5 ± 6
53	11	0	0	0	0	0	0	0	0	0	0	2	20	34	27	9	1				69.2 ± 9
65	12	0	2	10	17	17	19	17	8	0	1										24.9 ± 8
65	13	0	0	0	6	11	19	17	10	5	2										42.2 ± 10

TABLE IV

Mean RP values of single cones through 65 hours in darkness, based upon serial tangential sections. The eye at 0 hr. was light-adapted. Sections progress from the ELM toward the lamina basalis. Plus and minus values represent one standard deviation

Hours in DD	Fish number	Serial section sequence																				RP value
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
0	1	4	45	24	3																	19.8 ± 4
5	2	0	0	0	0	0	0	0	1	4	7	15	22	9	1							78.7 ± 9
5	3	0	0	0	3	3	5	6	20	29	27	1										62.7 ±13
17	4	0	0	3	25	25	9	19	20	23	23	25	26	29	9	4	5					45.5 ±19
17	5	2	6	7	12	13	4	11	16	18	10	2										32.0 ±13
29	6	0	0	0	0	1	2	1	6	1	2	5	11	16	15	15	1					63.2 ±14
29	7	0	0	0	0	1	3	4	1	1	0	0	7	33	25	13	11	1				60.4 ±10
41	8	0	0	6	14	14	6	5	5	9	17	18	11	1								46.8 ±14
41	9	0	1	1	5	9	11	10	4	5	10	12	13	20	29	23	22	15				58.6 ±19
53	10	0	0	0	0	0	1	2	4	4	8	16	7	15	13	16	12	22	26	8	1	65.2 ±15
53	11	0	0	0	0	0	0	5	2	4	8	12	15	12	13	3						66.4 ±12
65	12	0	13	15	9	15	14	15	19	13	4											26.6 ±11
65	13	0	3	10	13	17	12	17	11	8	1											36.9 ±11

maximal RP values occurred on the first night. After the first night, the cones did not migrate the full distances. Note also that on the first night the maximal extension of single cones exceeded that of the double cones, and that subsequently the single cones did not extend as far as the double cones. In the statistical summary of group C (Tables III and IV), the data for fish numbered 4 through 10 strongly suggested the existence of two populations of single cones. (We did not attempt to distinguish between types of single cones.)

The persistent rhythm in the cones was again demonstrated by group D (Figs. 6 and 7). Although the data for group A were incomplete for these purposes, they were included in the figures with the broken line suggesting the location of the point that is missing at 29 hr.

During 8 days in darkness, as shown by eyes fixed at 1300 hr. (Table V), the cones remained in the intermediate positions corresponding to the positions of the

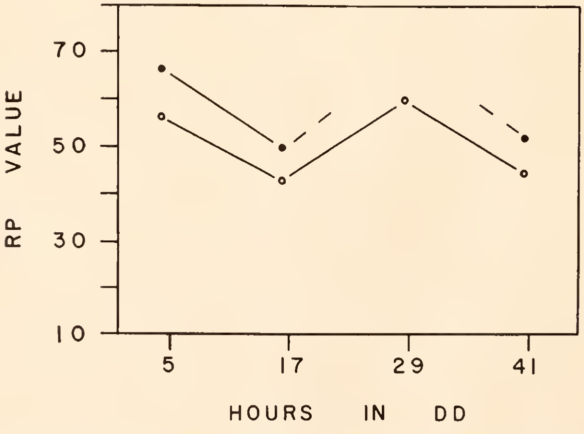

FIGURE 6. Rhythmic shift of positions of double cones based upon radial sections. ● Group A; ○ group D. Note group A no sample taken at 29 hours.

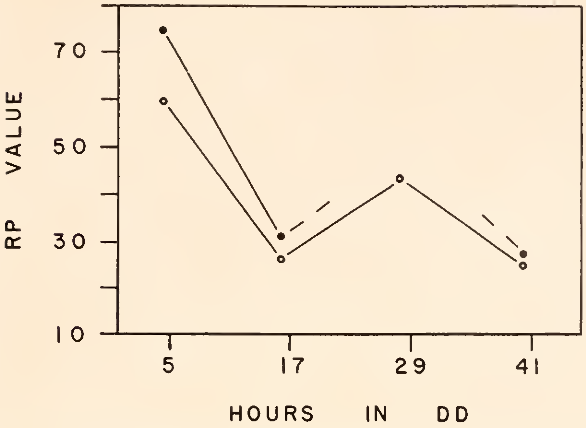

FIGURE 7. Rhythmic shift of positions of single cones based upon radial sections. ● Group A, ○ group D. Note group A no sample taken at 29 hours.

first two or three days. The RP values became consistently smaller for the single cones after the fourth day, but varied up and down for the double cones.

DISCUSSION

Although the retinas of different goldfish under the same conditions showed some marked variations, mean values based upon two fish for each sampling period adequately demonstrated the persistent circadian rhythm in the cones. There may be no statistically significant differences between consecutive points based upon small samples, but the probability of generating a cyclical function by chance is so small that there is little doubt of the validity of the rhythm in the goldfish. If we assumed a normal distribution for the variations between fish, the chance that the values for two fish would both fall either above or below the center of the distribution would be 1/4. The probability of getting the cycle over three days with

TABLE V

Mean RP values of single and double cones at 1300 hr. during 8 consecutive days in darkness. Plus and minus values represent one standard deviation

Day	Number of fish	RP value	
		Single cones	Double cones
1	4	31.3 ± 7.8	50.2 ± 3.9
2	2	28.3 ± 2.7	51.2 ± 0.5
3	3	33.9 ± 4.5	57.2 ± 3.7
4	1	30.6	59.5
5	1	29.2	46.9
6	2	22.8 ± 1.1	53.1 ± 3.6
7	1	19.2	31.7
8	2	20.1 ± 8.9	53.6 ± 6.5

12 fish would be 1/4096, and over two days with 8 fish it would be 1/256. The reproducibility of the rhythm with different lots of fish further supported its validity.

Using small samples, one could not predict the exact time of occurrence of maximal amplitudes in the retinomotor cycle as shown by the graphs in Figures 1 and 2, but the ease of obtaining the cycle indicated that the variations through the middle of the night did not generally overlap with those of the day. The rhythms were demonstrated by small samples collected at the mid-points of the nocturnal and diurnal segments of the cycle. In fact, had only one fish been represented in each sample, a cyclical function would have been obtained most of the time. In only one of 12 fish, no. 9 (Tables III and IV), did the mean positions of the cones deviate sufficiently to obscure the existence of a rhythm. The failure of Wigger (1941) to observe a persistent retinomotor rhythm in the goldfish is inexplicable.

We do not know why the values for group D were consistently lower than those of group A (Figs. 6 and 7). The fish were the same size, obtained at the same time of year, and treated in the same way, but did represent different years. We do not know whether the fish had different experiences prior to our purchasing them. It is well established from work on other organisms that the circadian rhythms are sensitive to a wide range of factors (Aschoff, 1965).

None of the cycles in this study or in the literature represent an individual fish. They represent small samples and the variations show that the results from small samples have limited comparative value. They also represent estimates of the mean values for the populations from which the samples were drawn, but it could be misleading to generalize about population values from small samples. Wigger (1941) paid particular attention to the fact that the cones in darkness became maximally extended before 2400 hr. and contracted about half the distance to a stable position between 2400 hr. and 0400 hr. before contracting farther. Our results (Figs. 1 and 2) illustrate that the time of maximum extension of the cones for any individual or small number of fish might occur at any time from 2300 hr. to 0300 hr. It would be interesting to be able to follow the course of migration of cones in a single fish.

It is important to note that Wigger (1941) sampled fish every two hours while we sampled fish every hour. If the graphs in Figures 1 and 2 were redrawn connecting points at two-hour intervals beginning at 2200 hr., the conspicuous fluctuations would disappear and the curves would become relatively smooth and symmetrical and would contradict our results as well as those of Wigger (1941). It is apparent that the shape of the curve is a function of the sampling interval.

The mean values of the graphs in Figures 1 and 2 suggest a change in the slope of the curve at 0300 hr. This change would be attributed to groups A and E, but the contrary results of group F suggest that the deflections of groups A and E were caused by chance. Further work would be required to determine the mean values and the nature of the variations for the population.

Engström (1960) described two types of single cones in the light-adapted goldfish retina and stated that the shorter type had no myoid process. This means that the shorter cones would not migrate during adaptive changes in the retina. We do not doubt the occurrence of two types of single cones, but a comparison of measurements from light-adapted (no. 1) and dark-adapted (nos. 2 and 3) fish in Table IV shows that all single cones migrated an average of 50 RP units, which means that all cones possessed myoid processes. We think that any interpretation of migratory capacities of visual cells should be based on a comparison of light- and dark-adapted eyes.

Our studies have shown that to obtain an eye in a state of maximal dark-adaptation, one must choose eyes during the first night of darkness. Thereafter, though the circadian rhythm would give a more dark-adapted eye at night than during noon darkness, the RP values would be distinctly lower than they would be on the first night. Arey and Mundt (1941) stated that the rhythm in the black bullhead, *Ameiurus nebulosus,* persisted through 4 days of constant darkness, the limit of their experiment.

Engström and Rosstrop (1963) interpreted this as a general guideline for experimental designs. To assure that the eyes of the roach, *Leuciscus rutilus,* were totally dark-adapted and free from the influence of a rhythm, they held the fish in darkness for 4 days before initiating studies on retinal adaptation at low levels of illumination. They illustrated the retina of a roach exposed to 10^{-6} ft. c. and stated (p. 155) that, "A histological comparison between eyes from '. . . 10^{-6} ft. c. . . .' and the totally dark-adapted ones does not reveal any noticeable differences." The dark-adapted eye was not identified, but was presumably one that had been in darkness for 4 days. They also did not state the time of fixing the eye. Their illustration of the retina of the roach looks like a 1300 hr. dark-adapted goldfish retina and not like a 2400 hr. dark-adapted retina. See Figure 3 and Tables III and IV for the relative positions of single and double cones in the 1200 hr. and 2400 hr. goldfish retinas. We think that the retina of the roach, after 4 days in darkness, was exhibiting a persistent rhythm. The question on the longevity of the persistent retinomotor rhythm in fishes has not been answered. At the end of three days, it was well defined in the goldfish, and the conditions of the retinas at 1300 hr. over a period of 8 days of darkness suggest that the rhythm was persisting. At least, the retina was remaining in an intermediate condition, not a dark-adapted condition.

Generalizations on the presence or absence of persistent retinomotor rhythms in fishes appear to be based on inadequate factual support. Von Studnitz (1952), citing the appropriate literature, mentioned two species in the discussion of rhythms, the black bullhead and the goldfish. Since, according to Wigger (1941) the goldfish did not show a persistent rhythm, the sole evidence for such a rhythm rested on the bullhead. Yet, Ali (1961) cited von Studnitz (1952) for the statement that rhythms occur in certain fishes. Engström and Rosstrop (1963) cited von Studnitz (1940) as the authority for the statement that persistent retinomotor rhythms are not general among fishes. There has not been sufficient study to support any wide generalization on persistent retinomotor rhythm in fishes, but the following quotation (p. 357) from Welsh and Osborn (1937) indicates that such rhythms may be widespread: "Several species (not named in the paper) other than *Ameiurus* were treated. . . . Not enough individual fishes were employed, however, to yield quantitative results, but without question, the phenomenon is fairly widespread."

SUMMARY

1. The goldfish retina shows a persistent circadian rhythm. In constant darkness for three days, the cones continued to shift positions in synchrony with the

diel cycle. The amplitude of the shift decreased after the first night. A maximally dark-adapted retina was obtained only on the first night.

2. Individual fish showed considerable variation in the time of occurrence of maximal dark-adaptation. This condition might be attained at any time between 2300 hr. and 0200 hr. The mean values for all fish suggested that the curve of progress of dark-adaptation for the population would be symmetrical with the maximal dark-adapted condition occurring at mid-night.

3. The single and double cones showed some characteristic differences in their behaviors. All cones migrated, but the relative excursions of single and double cones changed after the first night. The migratory patterns suggested the existence of two kinds of single cones.

4. The longevity of persistent rhythms in fish retinas is not known, but the assumption that it ceases after 4 days is based upon misinterpretation of a statement by Arey and Mundt (1941) about the black bullhead.

5. After 8 days in darkness the retina at 1300 hr. was in an intermediate state, not dark-adapted. If anything it had drifted toward the light-adapted state.

LITERATURE CITED

Ali, M. A., 1961. Histophysiological studies on the juvenile Atlantic salmon (*Salmon salar*) retina. II. Responses to light intensities, wavelengths, temperatures, and continuous light or dark. *Canadian J. Zool.*, **39**: 511–526.

Ali, M. A., 1963. Correlation of some retinal and morphological measurements from the Atlantic salmon (*Salmo salar*). *Growth*, **27**: 57–76.

Arey, L. B., and G. H. Mundt, 1941. A persistent diurnal rhythm in visual cones. *Anat. Record*, **79** (Suppl.), 5 (Abstr.).

Aschoff, J., 1965. Circadian Clocks. North-Holland Publ. Co. Amsterdam.

Engström, K., 1960. Cone types and cone arrangement in the retina of some cyprinids. *Acta Zool.*, **41**: 277–295.

Engström, K., and E. Rosstrop, 1963. Photomechanical responses in different cone types of *Leuciscus rutilus*. *Acta Zool.*, **44**: 145–160.

Nicol, J. A. C., 1965. Retinomotor changes in flatfishes. *J. Fish. Res. Bd. Canada*, **22**: 513–516.

Studnitz, G. von, 1940. Physiologie des Sehens. Retinale Primärprocesse. Akademische Verlagsgesellsschaft. Leipzig.

Studnitz, G. von, 1952. Physiologie des Sehens. Retinale Primärprocesse. 2nd ed. Akademische Verlagsgesllschaft. Leipzig.

Walls, G. L., 1942. The Vertebrate Eye and its Adaptive Radiation. Hafner Publ. Co. New York.

Welsh, J. H., and C. M. Osborn, 1937. Diurnal changes in the retina of the catfish, *Ameiurus nebulosus*. *J. Comp. Neurol.*, **66**: 349–359.

Wigger, H., 1941. Diskontinuität und Tagesrhythmik in der Dunkelwanderung retinaler Elemente. *Zeitschr. vergl. Physiol.*, **28**: 421–427.

CHANGES IN THE HEMOCYTE PICTURE OF GALLERIA MELLONELLA (LINNAEUS)[1]

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In this paper differential and total hemocyte counts were obtained and combined with hemolymph volume determinations in order to estimate the changes which occur in the hemocyte picture of the wax moth *Galleria mellonella* (Linnaeus) from the eleventh through the twenty-first days of larval life, during which period the larvae pass through successive phases of feeding, crawling, spinning a cocoon, and preparing to pupate.

When reared by the method of Beck (1960), the larvae reach a fairly large size within about 10 to 11 days. During the next 10 days or so they are particularly suitable for hematological studies. Hemolymph for differential counts was collected from manually immobilized, unanesthetized, 10- to 12-day-old larvae by piercing an intersegmental membrane with a sharp needle. Hemolymph for differentials from larger larvae was conveniently obtained either by cutting a proleg or one of the protuberances on the last abdominal segment. The fresh, unfixed, and undiluted hemolymph was collected directly on a slide and a coverslip added. The cells were examined with a phase contrast microscope at × 970 and were classified using the nomenclature of Jones (1962). From 200 to 1000 cells were identified per preparation. All studies were made on larvae freshly taken from an incubator held at 34° to 35° C.

Total hemocyte counts (cells per microliter) were generally made on the first drop of hemolymph emerging from a cut proleg of both unfixed (= untreated) and heat-fixed larvae.[3] Heat-fixation consisted of immersing larvae in a water bath at 55° C. for one minute. Hemolymph was quickly drawn to the 0.25 mark of a Thoma WBC diluting pipette and then rapidly diluted with 2% acetic acid to the 11 mark. After shaking vigorously and discarding the first three drops from the pipette, a double-lined hemocytometer was filled and the cells within 5 of the one-millimeter ruled squares were counted.

Hemolymph volumes were determined using the method of Yeager and Munson (1950), that is, by injecting the larvae with 10 microliters of 1% amaranth red in saline per gram body weight. Five larvae were used for each day of study. The dye was allowed to circulate for 3 to 5 minutes and a proleg severed and the hemolymph collected in a capillary tube. The intensity of the color was compared to a series of known dilutions of the dye. The hemolymph volume percentages were converted into microliters.

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[1] This research was sponsored by N.I.H. Grant HE 5193 and by Department Award K 3 GM 21,529. Scientific Article Number A1304, Contribution Number 3846 of the Maryland Agricultural Experiment Station.

[2] I am most indebted to Mrs. Daisy P. Liu, Mr. Richard A. Werner, and Dr. Ronald E. Wheeler for their assistance with various portions of this work. The manuscript was much improved by the comments of Drs. Malcolm Lea, Yasukito Nittono, and Gertraude Wittig and I am very grateful to them for their help.

[3] Differential and total counts were made on separately reared batches of larvae.

RESULTS

1. *Differential hemocyte counts*

During the last 10 to 12 days of larval life the following types of hemocytes could be easily recognized in unfixed hemolymph examined with phase microscopy: (1) prohemocytes, (2) plasmatocytes, (3) spherule cells, (4) adipohemocytes, and (5) oenocytoids, as Ashhurst and Richards (1964) have previously noted. Because so many transitional forms were seen between prohemocytes and plasmatocytes, it was very difficult or impossible to separate them accurately for quantitative work and these two types were combined into a common category which will, for convenience, be termed "plasmatocytoids." Cells seemingly transitional between plasmatocytoids and mature adipohemocytes were encountered during a definite period of larval life, and a series of counts were made in which this apparently intermediate category of cells was enumerated in addition to the other categories. These intermediate cells are termed *immature adipohemocytes.* Mitotically dividing hemocytes (probably prohemocytes) were counted and treated separately from the other categories. Adipohemocytes and spherule cells were never seen in division. The following types of hemocytes were not seen in *Galleria mellonella* larvae: granular hemocytes, cystocytes, podocytes, and vermiform cells. A few degenerating and unidentifiable hemocytes were encountered and they were so categorized in many differential counts.

Differential counts are given in Table I. During the actively feeding period (that is, from the eleventh through the fifteenth days of larval life), it is evident that (1) the plasmatocytoids ranged from 90% to 100% (with an overall mean of 96.7%), (2) adipohemocytes were consistently absent from the circulating hemolymph, (3) spherule cells varied from none to 7% and averaged 1.4%, (4) oenocytoids ranged from none to 8% and averaged 1.7%, (5) degenerating and unidentifiable hemocytes varied from none to 3%, and (6) mitotically dividing hemocytes (prohemocytes?) averaged 0.65%.

During the crawling, non-feeding, pre-cocoon-spinning period (approximately between the sixteenth and seventeenth days), a few adipohemocytes were noted in differential counts, and the number of dividing cells in such counts was reduced to about one-half that of the actively feeding period.

As soon as the larvae start to spin their cocoons, however, immature adipohemocytes suddenly increased to 12.6% and reached a maximum of 15.6% in lightly cocooned larvae and thereafter declined. Mature adipohemocytes steadily increased from 16% in the spinning period to a maximum of 57.1% in the newly formed pupae. Spherule cells rapidly declined, following the lightly cocooned period and were not observed in the pupae examined. Very few oenocytoids were seen in young pupae.

According to the differential counts, as the larvae transform into pupae, (1) plasmatocytoids decrease from about 96% to 41%, (2) immature adipohemocytes suddenly appear, (3) mature adipohemocytes steadily increase, (4) spherule cells

TABLE I

Differential hemocyte counts from unfixed last stage Galleria mellonella larvae and newly formed pupae. P = prohemocytes plus plasmatocytes (=plasmatocytoids); I = immature adipohemocytes; A = mature adipohemocytes; S = spherule cells; O = oenocytoids; D = degenerating hemocytes; U = unidentifiable hemocytes; M = mitotically dividing cells

Status	Number used	Days old	Number cells counted	Differential counts with ranges and means in parentheses (%)							
				P	I	A	S	O	D	U	M
Feeding in the medium	4	11	800	91–98 (95)	0	0	1–2 (1.4)	0–7 (2.5)	0–3	0–1	0–9 (2.5)
	4	12	1900	90–99 (96.8)	0	0	0–3 (1.4)	0–8 (1.5)	0–1	0–2	0–1 (0.2)
	5	13	2500	91–99 (95.4)	0	0	0–4 (1.4)	0–8 (2.9)	0–1	0	0–2 (0.5)
	10	13	2000	92–100 (96.5)	0	0	0–7 (2.4)	0–5 (1.1)	—	—	0–1 (0.25)
	5	14	1100	95–99 (97.2)	0	0	0–3 (1.3)	0–5 (1.2)	0–1	0–1	0–2 (0.7)
	10	14	2000	93–100 (98.1)	0	0	0–3 (0.6)	0–4 (1.3)	—	—	0–2 (0.3)
	5	15	2400	91–99 (96.5)	0	0	0–6 (1.5)	0–5 (1.8)	0–2	0	0–2 (0.3)
	10	15	2000	96–100 (98.0)	0	0	0–3 (1.0)	0–3 (1.0)	—	—	0–2 (0.45)
	5	16	1000	87–99 (95.5)	0	present	0–8 (2.7)	0–5 (1.7)	0–1	0–1	0–1 (0.2)
	10	16	2000	97–100 (98.4)	0	0	0–3 (1.0)	0–1 (0.6)	—	—	0–1 (0.25)
	5	17	1000	67–100 (92.7)	—	0–30 (4.8)	0–2 (0.5)	0–4 (1.9)	0	0–1	0–2 (0.6)
Spinning	10	17	2000	44–75 (59.0)	25–47 (37.9)	0–7 (0.9)	0–3 (0.9)	0–3 (1.3)	—	—	0–2 (0.55)
Early spinners	5	18	1000	45–80 (64.9)	—	15–47 (32.0)	0–6 (1.4)	0–3 (1.6)	0–1	0	0
Older spinners	5	19	1000	41–100 (80.0)	—	0–47 (15.1)	0–5 (2.2)	0–7 (2.7)	—	—	0–1 (0.3)
Lightly cocooned	3	20	600	31–95 (76.5)	—	0–68 (19.5)	0–2 (1.7)	0–4 (2.3)	0	0	0–1 (0.3)
	10	18	2000	20–52 (38.3)	35–57 (46.7)	0–42 (13.1)	0–3 (0.6)	0–3 (1.3)	—	—	0–1 (0.3)
	4	21	800	27–62 (41.0)	—	32–71 (54.9)	1–5 (2.3)	0–6 (1.7)	0–1	0	0
Densely cocooned	10	19	2000	29–61 (44.3)	2–57 (25.1)	1–56 (28.4)	0–4 (0.8)	0–6 (1.4)	—	—	0–1 (0.1)
	10	20	2000	31–63 (43.9)	0–19 (7.2)	29–62 (46.6)	0–3 (1.0)	0–3 (1.3)	—	—	0–1 (0.05)
	6	19–21	1200	18–47 (34.7)	—	51–79 (63.0)	0–2 (0.6)	0–5 (1.7)	0	0	0–1 (0.08)
Newly formed pupae	10	21	2000	21–66 (42.4)	0–3 (0.4)	34–79 (57.1)	0	0–1 (0.1)	—	—	0

decrease after larvae are lightly cocooned, (5) oenocytoids decrease, and (6) dividing cells steadily decline.

Out of 56 cases where records were kept, 75% of the dividing hemocytes were in metaphase, 23.2% in telophase, and 1.8% were in anaphase. Prophases could not be recognized with the methods used.

2. *Total hemocyte counts*

Total hemocyte counts (THC) were made daily from the thirteenth through the twenty-second days of life, from 150 unfixed and from 139 heat-fixed larvae. As reported in Table II, unfixed THC are more variable (4.9- *versus* 3.2-fold mean variation), and were consistently and significantly lower than heat-fixed counts at greater than the 95% level (that is, twice the standard errors of daily unfixed and heat-fixed counts did not approach an overlap on any of the days studied). In both types of counts the numbers of cells per microliter of hemolymph increase as larvae proceed toward the pupal stadium, and in both the increase is about the same (*i.e.*, from 17,000 to 18,000 hemocytes/microliter). In both, the most variable counts were obtained on the seventeenth day. The unfixed counts from 20- to 22-day-old larvae are comparable to the value of 33,200 hemocytes per microliter reported by Stephens (1963) for larvae which were reared by a different method and developed much more slowly. However, the consistently and significantly higher counts from heat-fixed *Galleria* are definitely not in agreement with Stephens' statement that heat fixation does not alter the counts.

Table II

Daily total hemocyte counts with standard errors from unfixed and heat-fixed Galleria mellonella larvae

Days old	No. used	Cells per microliter		
		Unfixed	No. used	Heat-fixed
13	5	20,336 ± 3360	5	50,448 ± 4640
14	11	21,933 ± 1804	10	49,686 ± 3200
15	16	23,704 ± 2210	10	42,992 ± 3480
16	16	31,045 ± 2635	15	53,504 ± 1661
17	22	25,252 ± 1531	22	43,316 ± 2869
18	16	27,609 ± 1880	16	54,042 ± 4425
19	17	27,053 ± 2334	16	56,432 ± 5460
20	15	31,925 ± 2779	15	48,724 ± 3739
21	16	37,744 ± 2320	15	61,752 ± 3515
22	16	35,613 ± 3545	15	54,672 ± 3488
	Mean	28,221.4 ± 2440	Mean	51,556.8 ± 3648

Unfortunately only a few records were made on the status of the various larvae during the above period. The records which were obtained, however, were as follows. In 6 newly emerged larvae the unfixed THC averaged 22,666. During the spinning of the cocoon, 32,584 cells were found per microliter from 10 unfixed larvae. Three larvae from light cocoons had 33,067 cells per microliter. Unfixed

THC from four larvae taken from dense cocoons amounted to 44,620 cells, and counts from three prepupae came to 17,867 hemocytes. These few data suggest that the THC increases as larvae spin their cocoons, that there is a further and greater increase after they complete the cocoon-spinning process, and that the counts begin to decrease in the prepupae. The counts from densely cocooned larvae and pharate pupae are strikingly higher than those found by Shrivastava and Richards (1965), possibly because they used chilled material and excluded the first two drops of hemolymph.

3. *Hemolymph volumes*

Hemolymph volume determinations were made from 15- to 22-day-old unfixed *Galleria,* all from a single batch of individuals. The larvae were all still feeding in the medium on the seventeenth day. On the eighteenth day, four out of 5 larvae had already spun a light cocoon. By the twenty-second day, they had all pupated. As presented in Table III, the hemolymph volumes when viewed as percentages of the body weight remain level at about 34% during the feeding period and then gradually decrease to less than 16.4% in newly formed pupae. Considered as microliters, hemolymph volumes of feeding larvae averaged 56.7 and tended to decline thereafter. Hemolymph volumes of cocooning or cocooned larvae averaged 45.7 microliters. With pupation, the volumes obtained were from less than 17 to 24.5, with a mean of about 19 microliters.

TABLE III

Hemolymph volumes of 15- to 22-day-old Galleria mellonella (unfixed)

Age in days	Status	% Body weight		Calculated microliters	
		Range	Mean	Range	Mean
15	Feeding	33–36	34.2	36.5–54.0	43.1
16	Feeding	33–36	33.6	63.2–73.6	67.7
17	Feeding	33–36	34.2	48.1–72.2	59.4
18	Lightly cocooned*	28–35	31.6	40.1–53.7	46.7
19	Medium cocoon	29–33	31.4	41.5–7.16	58.1
20	Dense cocoon	19–31	26.0	29.9–57.7	42.4
21	Dense cocoon	16–32	24.8	27.8–46.3	35.8
22	Pupae	16–18	16.4	17.0–24.5	18.8

* Only one of the larvae was beginning to spin a cocoon.

Hemolymph volumes were also made from a subsequent batch of 10 unfixed and 10 heat-fixed larvae of the same age. The hemolymph volumes were identical (45.5 microliters).

4. *Calculated hemocyte populations*

The preceding information can be combined to indicate changes in the hemocyte population within the entire insect. Thus, when THC values are multiplied by the hemolymph volumes, it can be calculated that, at the 95% level, there are from 831,140 to 2,458,525 hemocytes available in the circulating hemolymph of unfixed larvae from the fifteenth through the twenty-first days, with means fluctuating around 1,456,000 (Table IV). The daily mean hemocyte population from the fifteenth through the twenty-first days varied by a factor of only 1.2-fold. The data suggest that the circulating hemocyte population in unfixed larvae remains about the same up to pupation itself, at which time there is a very striking and significant decrease (at the 95% level) so that more than one-half of the hemocyte population is no longer circulating in unfixed newly formed pupae.

5. Calculated changes in the components of the hemocyte population

Calculations on the components of the hemocyte population from the fifteenth through the twenty-second days are presented in Tables V and VI. Assuming that these data give a reasonable approximation towards the real situation, the following estimations can be made. (1) During the feeding period, plasmatocytoids averaged 1,490,569 (95% range = 802,050–2,419,189) and during cocooning they averaged 563,308 (95% range = 411,238–816,445), amounting to an average decrease of 927,261 cells (95% range = 390,812–1,602,744). With pupation, there was a further average loss of 185,000 cells (95% range = 183,876–186,125), amounting to an average total loss of 1,112,261 plasmatocytoids (95% range = 574,688–1,788,869) as *Galleria* larvae transform into pupae. (2) From 520,000 to 684,123 immature and 145,900 to 192,000 matured adipohemocytes appeared in the hemolymph of lightly cocooned larvae, a range of 666,000 to 876,000 (average 771,025) cells containing lipid inclusions. Immature adipohemocytes decreased by 439,627–569,695 (average 504,661) cells within the densely cocooned larval insect. With pupation, there was a further decrease of 78,348–111,217 cells (average 184,996), thus amounting to 517,975–680,912 immature adipohemocytes deleted during the larval-pupal molt and ecdysis. (3) After mature adipohemocytes first appeared in the hemolymph, they increase to a maximum of 955,929. Between the eighteenth and twenty-first days, 600,726–764,023 (average 682,375) mature adipohemocytes were formed, on each of which days they increased by 151,000–332,000. After pupation, 440,000–498,000 adipohemocytes were no longer circulating. (4) During larval life, spherule cells averaged 16,950 and they were not observed in the pupae examined. They reached a maximum on the sixteenth day, that is, before the

TABLE IV

Calculated circulating hemocyte populations in unfixed 15- to 22-day-old Galleria mellonella

Age in days	Status	Range at 95% level	Mean
15	Feeding	831,140–1,212,144	1,021,642
16	Feeding	1,744,967–2,458,525	2,101,746
17	Feeding	1,318,086–1,681,852	1,499,969
18	Light cocoon	1,113,748–1,464,932	1,289,340
19	Medium cocoon	1,300,568–1,842,990	1,571,779
20	Dense cocoon	1,117,961–1,589,279	1,353,620
21	Dense cocoon	1,185,123–1,517,347	1,351,235
22	Pupae	536,232– 802,816	669,524

TABLE V

Calculated mean changes in the components of the hemocyte population of unfixed 15- through 22-day-old Galleria mellonella

Age in days and status	Numbers of circulating hemocytes					
	Plasmatocytoids	Adipohemocytes		Spherule cells	Oenocytoids	Dividing hemocytes
		Immature	Mature			
15 Feeding	985,884	0	0	15,325	18,389	3,065
15 Feeding	1,001,209	0	0	10,012	10,012	4,597
16 Feeding	2,007,167	0	0	56,747	35,730	4,203
16 Feeding	2,068,118	0	0	21,017	12,610	5,254
17 Feeding	1,390,471	0	71,998	7,499	28,499	9,000
18 Light cocoon	493,817	602,122	168,903	7,736	16,761	3,868
19 Dense cocoon	696,298	394,516	446,385	12,574	22,005	1,572
20 Dense cocoon	594,239	97,461	630,787	13,536	17,597	667
21 Dense cocoon	468,878	—	851,278	8,107	22,971	1,081
22 Pupae	283,878	2,678	382,298	0	669	0

larvae began to spin a cocoon. (5) Oenocytoids fluctuated from 10,012 to 35,730 during larval life, with an overall mean of 20,508 and, like the spherule cells, attained a maximum on the sixteenth day. (6) From 559 to 10,091 hemocytes apparently divide in the hemolymph from the fifteenth through the twenty-first days of life, the greatest number being present on the seventeenth day. Since it is not known whether mitotic divisions occur throughout the day and since the duration of the mitotic cycle is unknown, it is not possible to make correlations between mitoses and changes in the hemocyte population.

In their radioautographic study, Shrivastava and Richards (1965) showed that plasmatocytes of *Galleria* transform into adipohemocytes within 24 hours, and the present hemocyte population calculations were examined to see if the changes in the population of plasmatocytoids could be correlated with adipohemocyte popula-

TABLE VI

Calculated ranges at 95% level of plasmatocytoids and adipohemocytes

Age in days and status	Plasmatocytoids	Adipohemocytes	
		Immature	Mature
15 Feeding	802,050–1,169,719	0	0
15 Feeding	814,517–1,187,901	0	0
16 Feeding	1,666,443–2,347,891	0	0
16 Feeding	1,717,047–2,419,189	0	0
17 Feeding	1,221,866–1,559,077	0	58,170– 74,836
18 Light cocoon	426,565– 561,069	520,120–684,123	145,901–191,906
19 Dense cocoon	576,152– 816,445	326,443–462,590	369,361–523,409
20 Dense cocoon	490,785– 697,693	80,493–114,428	520,970–740,604
21 Dense cocoon	411,238– 526,519		746,627–955,929
22 Pupae	227,362– 340,394	2,145– 3,211	306,188–458,408

tion changes. The data in Table VII show the changes in the populations of plasmatocytoids and of immature and mature adipohemocytes in terms of the 95% ranges with the means in parentheses. (1) There is no correlation of changes in the two populations between the sixteenth and seventeenth days (that is, 7.5 to 10.5 times more plasmatocytoids disappear than adipohemocytes appear). (2) If it is assumed that all of the mature adipohemocytes already in circulation on the seventeenth day (71,998 cells) remain in circulation on the eighteenth day, then 96,905 new adipohemocytes would need to be formed from plasmatocytoids. Between the seventeenth and eighteenth days, 896,654 plasmatocytoids disappeared and 771,025 adipohemocytes appeared which leaves a deficit of 125,629 plasmatocytoids unaccounted for in a population of 1,264,842 (an error of 10%). This is interpreted to mean that many (about 64%) plasmatocytoids transform into immature and mature adipohemocytes between the seventeenth and eighteenth days. (3) Between the eighteenth and nineteenth days the plasmatocytoids increased by 202,481 cells, the immature adipohemocytes decreased by 207,606 cells, while mature adipohemocytes increased by 277,482 cells. If all of the mature adipohemocytes of the eighteenth day remained in circulation on the nineteenth day, then 277,482 new mature adipohemocytes would need to be formed. If all 207,606 immature adipohemocytes which disappeared from the circulation between the eighteenth and nineteenth days were transformed into mature adipohemocytes, this would leave only 69,876 mature adipohemocytes unaccounted for on the nineteenth day. This appears to be an excellent correlation and implies that about 34% of the 168,903 immature adipohemocytes in circulation on the eighteenth day transform into mature cells by the nineteenth day. (4) Between the nineteenth and twentieth days the populations of both plasmatocytoids and immature adipohemocytes appear to decrease simultaneously and far more immature adipohemocytes disappear than new ones form. No correlations could be detected then between the various hemocytes between the nineteenth and twentieth days. (5) Between the twentieth and twenty-first days the plasmatocytoids decreased by 125,361 cells and mature adipohemocytes increased by 220,491 cells. If all 530,787 mature adipohemocytes of the twentieth day remained in circulation and all of the circulating immature adipohemocytes transformed into mature cells by the twenty-first day, this would still leave 123,030 mature adipohemocytes unaccounted for. If most of the 125,361 plasmatocytoids which disappeared between the twentieth and twenty-first days transformed into

TABLE VII

Estimated increases and/or decreases in populations of plasmatocytoids, immature and mature adipohemocytes in unfixed Galleria mellonella. Ranges at 95% level; means in parentheses

Between days	Plasmatocytoids	Adipohemocytes	
		Immature	Mature
16–17	−495,181 to 860,112 (−677,647)	—	+ 58,170 to 74,836 (+ 71,998)
17–18	−795,301 to 998,008 (−896,654)	+520,120 to 684,123 (+602,122)	+ 87,731 to 117,070 (+ 96,905)
18–19	+149,587 to 255,376 (+202,481)	−193,677 to 221,533 (−207,606)	+223,460 to 331,503 (+277,482)
19–20	− 85,367 to 118,752 (−102,059)	−245,950 to 348,162 (−297,055)	+151,609 to 217,195 (+184,402)
20–21	− 79,547 to 171,174 (−125,361)	—	+215,325 to 225,657 (+220,491)

mature adipohemocytes, this would account for the deficit and make an almost perfect correlation.

Considering the many sources of error in calculations such as these, it is remarkable that it was possible to make any correlations, and impressive that three out of five of them appear so close. These correlations suggest (1) that many plasmatocytes transform into both immature and mature adipohemocytes between the seventeenth and eighteenth days when larvae are spinning a cocoon, (2) that between the eighteenth and nineteenth days, when the larvae are cocooned, mature adipohemocytes are largely being formed by maturation of immature adipohemocytes, and (3) that in pharate pupae mature new adipohemocytes are being formed from both immature adipohemocytes and from plasmatocytoids between the twentieth and twenty-first days.

Discussion

In *Prodenia* larvae Yeager (1945) recognized and counted separately adipohemocytes (his "spheroidocytes") and granular hemocytes (his "cystocytes"). Jones (1959) pointed out that when the adipohemocytes of *Prodenia* matured they closely resemble the granular hemocytes. Yeager (1945) suggested that the adipohemocytes were derived from prohemocytes and, since he observed mitoses among adipohemocytes, they might also be considered as a self-perpetuating line of cells in this insect. He suggested that the granular hemocytes of *Prodenia* were derived from plasmatocytes. He observed mitoses among granular hemocytes, though less commonly than in the plasmatocytes and adipohemocytes. In *Bombyx,* Nittono (1960) apparently combined Yeager's cystocytes and spheroidocytes into a common category which he designated granular hemocytes. Earlier, Jones (1959) had suggested that Yeager's "cystocytes" were comparable to the granular hemocytes of other insects. In some insects, cells termed granular hemocytes are quite distinct from both plasmatocytes and adipohemocytes: for example, in the blood-sucking bug, *Rhodnius prolixus* (Jones, 1965), the granular hemocytes possess many uniform discrete inclusions and are not derived from plasmatocytes and are not related to them. In *Sarcophaga,* changes in the population of cells termed granular hemocytes (Jones, 1956) cannot be correlated with changes in the population of plasmatocytes (Jones, unpublished data). The "granular hemocytes" of *Bombyx* are present in large numbers in one- to three-day-old larvae of the fifth stage and they were frequently observed in mitotic division (p. 262) by Nittono. The data in Nittono's Tables 4 and 6 were combined so that estimates could be made of the components of the hemocyte population with time in both males and females of the fifth stage. No correlations at all could be found between the populations of plasmatocytoids and adipohemocytes at any time. Dr. Nittono (personal communication) has confirmed this. Can the granular hemocytes of *Bombyx* and *Sarcophaga* which are present throughout larval life and which do not appear to be derived from plasmatocytes be compared with the adipohemocytes of *Galleria* when the latter appear *only* near the end of larval life, do *not* divide, and *are* derived from plasmatocytes? Granular hemocytes and adipohemocytes may both be phagocytic and yet very different in their origins. There is no doubt that the granular hemocytes in *Rhodnius* are *not* comparable morphologically or physiologically to the adipohemocytes of *Galleria.* Until considerably more information is available concerning the granular hemocytes and adipohemocytes, separate terms should be retained. From the evidence now available it would seem that granular hemocytes of some insects are *not* derived from plasmatocytes whereas adipohemocytes of a number of the Lepidoptera arise by direct transformation of circulating plasmatocytes.

In *Prodenia,* plasmatocytoids decline from 86.3% in the first instar larvae to 34.2% in prepupae (calculations from Yeager's data, 1945). Granular hemocytes appeared first in fourth-stage larvae and increased to 28% just before pupation (Yeager, 1945). Adipohemocytes increased from 2.5% in first stage larvae to 38.6% in prepupae. Spherule cells reached their maximum (43.4%) in third-stage larvae and declined to 6% the day before pupation (Yeager, 1945). During the last three days before pupation of *Prodenia,* plasmatocytoids decreased from 41.7% to 17.8%, adipohemocytes increased from 33.9% to 43.9%, granular hemocytes increased from 5.2% to 28%, and oenocytoids decreased from 5.1% to 1.8% (Yeager, 1945).

In last-stage *Bombyx* larvae, plasmatocytoids reached a peak of 67.3% on the seventh day and declined to 27.3% just before pupation. In the J 122 X C strain, the granular hemocytes averaged 53.4% from the third through the fifth larval stages and definitely increased near the end of each stadium. During the first eight days of the last larval stage they averaged 45.7%, and during the last four days they averaged 64.1% (Nittono, 1960).

Galleria resembles *Prodenia* and *Bombyx* in that plasmatocytoids decrease and that hemocytes with many polysaccharide and/or lipid or other types of inclusions increase prior to pupation. *Galleria* differs significantly from *Prodenia* and *Bombyx* in that their hemocytes with many polysaccharide and/or lipid or other types of inclusions do not appear in the hemolymph for the first five days of the last larval stadium. The hemocytes with lipid inclusions in *Bombyx* apparently are not derived from plasmatocytes (at least no correlations between changes in these two components of the population were detectable), whereas in *Galleria* there is radioautographic evidence that hemocytes with lipid inclusions are derived from plasmatocytes, and in three out of five cases it was possible to detect reasonably close reciprocal correlations between the changes in these two cell types.

SUMMARY

1. The hemocytes of *Galleria mellonella* (Linnaeus) larvae were identified and differentially counted in unfixed hemolymph with phase microscopy. The numbers of hemocytes per microliter of hemolymph were obtained from both unfixed and heat-fixed larvae. Hemolymph volumes were determined by the amaranth red method. These studies were made to determine what changes in the hematology occur as the last stage larvae pass through distinctive phases in transforming into pupae.

2. In differential counts, plasmatocytoids decrease, immature adipohemocytes suddenly appear, and mature adipohemocytes steadily increase. Spherule cells, oenocytoids and dividing hemocytes decrease as *Galleria* larvae develop into pupae.

3. The numbers of hemocytes per microliter of hemolymph increase as *Galleria* larvae proceed towards the pupal stage in both unfixed and heat-fixed animals. Counts were always significantly higher in heat-fixed than in unfixed larvae.

4. The hemolymph volume is the same in both unfixed and heat-fixed larvae. The hemolymph volume declines from about 34% (56.7 microliters) in precocoon-spinning larvae to less than 16.4% (19 microliters) in newly formed pupae.

5. It is estimated from the various data presented that an average of 1,456,000 hemocytes remain in circulation within the hemocoele of unfixed larvae from the fifteenth through the twentieth days of life, and that with pupation more than one-half of these cells fall out of circulation.

6. In three out of 5 cases it was possible to correlate decreases in the plasmatocytoid population with increases in adipohemocytes. It is suggested that during the spinning of a cocoon plasmatocytoids transform into both immature and mature adipohemocytes, that when the larvae are densely cocooned mature adipohemocytes are largely formed by the maturation of immature adipohemocytes, and that in pharate pupae new mature adipohemocytes are derived from both immature adipohemocytes and plasmatocytoids.

7. The hemocyte picture of *Galleria* is compared to that of *Prodenia* and *Bombyx*. In all three of these Lepidoptera the plasmatocytoids decrease and the hemocytes with many polysaccharide and/or lipid or other types of inclusions increase prior to pupation. *Galleria* differs from the other two species in that their hemocytes with lipid or other inclusions do not appear until about the sixteenth or seventeenth days of larval life, do not divide, and in many cases are derived from circulating plasmatocytes.

LITERATURE CITED

Ashhurst, D. C., and A. G. Richards, 1964. Some histochemical observations on the blood cells of the wax moth, *Galleria mellonella* L. *J. Morph.*, **114**: 225–254.

Beck, S. D., 1960. Growth and development of the greater wax moth, *Galleria mellonella* (L.) (Lepidoptera: Galleriidae). *Wisconsin Acad. Sci., Arts and Letters*, **49**: 137–148.

Jones, J. C., 1956. The hemocytes of *Sarcophaga bullata* Parker. *J. Morph.*, **99**: 233–257.

Jones, J. C., 1959. A phase contrast study of the blood-cells in *Prodenia* larvae (Order Lepidoptera). *Quart. J. Micr. Sci.*, **100**: 17–23.

Jones, J. C., 1962. Current concepts concerning insect hemocytes. *Amer. Zool.*, **2**: 209–246.

Jones, J. C., 1965. The hemocytes of *Rhodnius prolixus* Stål. *Biol. Bull.*, **129**: 282–294.

Nittono, Y., 1960. Studies on the blood cells in the silkworm, *Bombyx mori* L. *Bull. Sericult. Exp. Sta.*, **16**: 171–266.

Shrivastava, S. C., and A. G. Richards, 1965. An autoradiographic study of the relation between hemocytes and connective tissue in the wax moth, *Galleria mellonella* L. *Biol. Bull.*, **128**: 337–345.

Stephens, J. M., 1963. Effects of active immunization on total hemocyte counts of larvae of *Galleria mellonella* (Linnaeus). *J. Ins. Path.*, **5**: 152–156.

Yeager, J. F., 1945. The blood picture of the southern armyworm (*Prodenia eridania*). *J. Agric. Res.*, **71**: 1–40.

Yeager, J. F., and S. C. Munson, 1950. Blood volume of the roach *Periplaneta americana* determined by several methods. *Arthropoda*, **1**: 255–265.

THE EFFECT OF LIGHT ON THE SPAWNING OF CIONA INTESTINALIS

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Invertebrate embryologists have long known that a number of ascidians spawn in response to light following darkness. *Molgula manhattensis* (Castle, 1896; Conklin, 1905) and *Ciona intestinalis* (Castle, 1896; Conklin, 1905; Berrill, 1947) normally spawn at dawn but can be induced to spawn at any time by keeping them in the dark until needed; then a short exposure to light causes them to spawn (Costello *et al.,* 1957). *Styela partita* spawns during the late afternoon (Castle, 1896; Conklin, 1905; Rose, 1939). Rose (1939) found that *S. partita* could be induced to spawn at any time by placing them in the dark for 12 hours, then subjecting them to light for 11–12 hours, at the end of which time they spawn.

The physical factors controlling spawning in *Corella parallelogramma* have been extensively investigated by Hŭus (1939, 1941a, 1941b). This ascidian, which normally spawns during the early morning, can be caused to spawn at any hour by exposing dark-adapted animals to the light of a 60-candle bulb 25 cm. from the aquarium for 2 minutes (Hŭus, 1939). Spawning begins within 30 minutes. Hŭus termed this period between illumination and spawning the "dormant period." Limiting temperatures for spawning were found to be 10°–24° C. (Hŭus, 1941a). The duration of the dormant period was determined to be temperature-dependent (1941b), 11 minutes being required at 24° C. and 17 minutes at 10.5° C. Hŭus hypothesized that light causes spawning by eliciting the production of some unknown hormone; the temperature dependency of the dormant period, he stated, tended to support this view.

The present study, on light-induced spawning by *C. intestinalis,* consists of two series of experiments. The first series, using unmeasured white light, determined the minimum reliable dark-adaption time and the time required for spawning after illumination. The second series, using quantified monochromatic light, determined the threshold dose of light energy necessary to cause spawning at different wave-lengths. From these data an action spectrum for spawning is constructed.

MATERIALS AND METHODS

Experimental animals

Ciona intestinalis between 4 and 7 cm. in overall length were collected in Mission Bay, San Diego, California. Only gravid individuals, identified by their full oviducts, were used in the experiments. Continuous illumination from the time of collection until the dark-adaption period prevented uncontrolled spawning. The animals were used only once, two days after collection.

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Experimental apparatus and procedure

The preliminary experiments used white light from two Sylvania 40-watt daylight fluorescent tubes 147 cm. above the animals. For these experiments, the animals, each in a 400-ml. beaker of sea water, were placed on a 15°–17° C. water table. Dark-adaption for periods of 45 to 60 minutes was accomplished by covering the water table. The animals were either continuously illuminated until spawning occurred, or they were returned to darkness after a one-minute exposure to light.

A Bausch and Lomb high intensity, grating monochrometer (1350 grooves/mm.), equipped with a tungsten (quartz-iodine) 45-watt lamp as light source, was used for monochromatic illumination. The band pass was 10 mμ. The beam from the exit slit (exit lens removed) passed through a leaf shutter, then through the end of the aquarium (10 cm. from monochrometer) to the animal. An approximately circular spot of light, 3 cm. in diameter, was formed on the animal by the exit beam at this distance.

The monochromatic light intensity was measured with phototube C of a Photovolt electronic photometer, model 501-M, placed in the same position relative to the monochrometer as had been the end of the aquarium. The photometer was calibrated at all wave-lengths used against a calibrated Reeder compensated vacuum thermopile (RBL-500) and a Leeds and Northrup (2284b) high sensitivity galvanometer. A National Bureau of Standards 50-watt 115v. secondary Radiometric Standard Lamp was used to calibrate the thermopile-galvanometer system according to the method contained in form NBS-443.

The animals were suspended with nylon string, basal ends upward, in individual 600-ml. beakers of sea water at 16°–17° C. After one hour of dark-adaption the animals were removed from their beakers, placed in the experimental aquarium and illuminated with monochromatic light, one at a time. While being illuminated, the animals were pressed flat against the aquarium end by a flat flask. Since the circle of illumination was only 3 cm. in diameter, only the siphonal end of the animals received the light. After illumination, the animals were returned to their beakers. The beakers were examined for ova 30 minutes after the last animal had been illuminated. Those beakers that contained ova were recorded as positive. Those that did not contain ova were brought into the well-lit laboratory (fluorescent lighting) for 30 minutes, after which time they were again examined for the presence of ova. Beakers that now contained ova but which had not on removal from the darkroom were recorded as negative, that is, the animals were capable of spawning but had not been provoked to spawn by the amount of light energy received.

The duration of exposure at the maximum intensity of a given wave-length necessary to provoke spawning in two out of three animals, when a 10% shorter exposure would not elicit spawning by two out of three animals, was taken as the threshold duration of illumination for spawning at that wave-length. This threshold duration was determined for wave-lengths between 400 mμ and 610 mμ in 15-mμ increments. These values were then converted to threshold doses in quanta/mm^2. An action spectrum was constructed by graphing the reciprocal of the threshold dose against wave-length.

RESULTS AND DISCUSSION

White light studies

Dark-adaption periods of 45–55 minutes followed by return to light resulted in spawning by 26 out of 65 animals (40%). One hour of dark-adaption preceding illumination elicited spawning in 47 of the 60 animals tested (78.3%). Following the one-hour dark-adaption period, an average of 27.3 minutes elapsed before spawning occurred. The one-hour dark-adaption period was used for all of the following experiments.

A comparison of these results with *Ciona* and Hŭus's with *Corella* demonstrates clearly that these two ascidians have quite similar spawning responses to light, the main difference being duration of the latent (dormant) period: at 14.5° C., *Corella* spawned 14.5 minutes after exposure to light; *Ciona* spawned 27 minutes after exposure at 15°–16° C.

The observation that *Ciona* spawns at dawn in the laboratory is an old one (Castle, 1896; Conklin, 1905; Berrill, 1947), yet the most recent paper on the spawning of this ascidian (Carlisle, 1951) curiously omits any reference to the light-induced spawning of any ascidian. Carlisle (1951) was investigating the spawning of *Ciona intestinalis* and *Phallusia mammilata* in relation to two other factors: the effect of injecting human chorionic gonadotropin and the effect of ingesting gametes. Carlisle, without discussing the illumination of his laboratory, stated that *Ciona* was never observed to spawn "spontaneously." We are quite confident that in spite of the small number of animals involved in his studies (less than 60), had his laboratory ever been darkened, spawning would have occurred, provided the animals were ripe. Carlisle reported that either injection of chorionic gonadotropin or ingestion of gametes provoked spawning in these two ascidians. This spawning took place 20 hours after treatment, in contrast to the 27-minute latent period established here. Carlisle further states that prior to treatment, no corpora lutea were observed in *Ciona*'s ovary. Millar's (1953) report that the oviduct is always packed with ova prior to spawning has been fully confirmed by our observations. Although the histological structure of the ovary was not examined in this study, the presence of ova in the oviduct implies that corpora lutea should be found in the ovary. A re-evaluation of Carlisle's findings may be made in the light of the observations reported here. Perhaps Carlisle did not provoke spawning by his treatments, but instead induced ovulation. These two phenomena, as demonstrated by the full oviduct prior to spawning, are quite separate in *Ciona*. It should be stated here that Hŭus (1941a) found that *Corella,* prior to spawning, has an empty oviduct, which suggests that in *Corella* spawning and ovulation are either simultaneous or occur closer in time than in *Ciona*.

Monochromatic light studies

The action spectrum for spawning of *C. intestinalis* was obtained by illuminating the animals at different wave-lengths and determining the threshold duration of exposure required to evoke spawning at the maximum intensity of each of these wave-lengths. Since the intensity of the incident beam at each wave-length was known, the quantum requirement (the threshold dose for spawning) was easily calculated.

Since the intensity at each wave-length was different, it is possible that the quantum requirement, determined on the basis of duration of exposure, might have been different if the Reciprocity Law does not hold for some intensities used. However, since the maximum difference in intensity between any two wave-lengths was less than four times (Table I), and since the animals were most sensitive to the wave-lengths showing the lowest intensity, this problem probably does not seriously influence the shape of the action spectrum. Another drawback to this method which became evident as the experiments progressed was that for a reasonable exposure time (5 minutes) the energy output of the monochrometer was too low in the red end of the spectrum to cause spawning. This fact also made it impossible to test for reciprocity, *i.e.,* Intensity × Time equals a Constant Response, at each wave-length used.

TABLE I

Experimental and derived data necessary to establish spawning threshold in quanta

Wave-length mμ	Intensity		Threshold	
	Ergs/sec./mm.^{2}	Quanta × 10^{12}/sec./mm.^{2}	Duration sec.	Dose Quanta × 10^{14}/mm.^{2}
610	17.64	5.41	660	35.7
595	18.59	5.56	498	27.7
580	14.14	4.12	360	14.9
565	14.62	4.15	88	3.67
550	15.54	4.30	44	1.89
535	14.30	3.86	72	2.77
520	14.00	3.66	66	2.42
505	10.92	2.77	216	6.05
490	13.26	3.27	577	18.9
475	11.52	2.76	378	10.4
460	10.12	2.34	570	13.4
445	8.28	1.86	478	8.89
430	9.90	2.07	56	1.16
415	4.80	1.00	60	0.60
400	4.86	0.978	144	1.41

Table I presents the raw and derived data necessary to obtain the threshold quanta requirements for spawning at all wave-lengths tested.

Of the 884 animals used in this study, 589 (66.6%) spawned in response to light, either after illumination by monochromatic light or after return to the illuminated laboratory.

Action spectrum for spawning

Figure 1 is an action spectrum for photically induced spawning by *Ciona intestinalis.* The reciprocals of the quantum thresholds from Table I are plotted against wave-length to show the relative effectiveness of light of each wave-length in inducing spawning. As can be seen from this figure, there are three peaks of maximum effectiveness. Wave-length 415 mμ is most effective, requiring a dose of light about one-third that of the next most effective wave-length, 550 mμ, to induce spawning. Wave-lengths 520 mμ and 550 mμ are of nearly equal effectiveness. This action spectrum for spawning by *Ciona intestinalis* suggests that a hemoprotein is the light-absorber because of the great efficiency in the region of the Soret band absorption and the characteristic peaks in the yellow. An examination of the absorption spectra of the hemoproteins led to cytochrome *c* as a possible chromophore.

In Figure 2 the action spectrum for spawning in *Ciona* is replotted as the Relative Effectiveness in Inducing Spawning as a function of wave-length. These data are obtained by setting the reciprocal of the threshold dose in quanta/mm.^{2} at wave-length 415 mμ equal to 100% Relative Effectiveness. The doses at all other wave-lengths are then reduced to a percentage of the dose at 415 mμ. On the same figure (Fig. 2) are plotted data on the Relative Optical Density of reduced horse heart cytochrome *c*. These data are calculated from those obtained by Margoliash and Frohwirt (1959) by setting the optical density at wave-length 415 mμ equal to 100% Relative Optical Density. At all other wave-lengths, the Relative Optical Density is calculated as a percentage of the optical density at 415 mμ. Comparison of these two curves shows that they are similar in many respects. Oxidation of cytochrome *c* results in the following changes in its absorption spectrum: the major peak at 415 mμ shifts to 410 mμ and is lowered considerably, and the peaks at 520 mμ and 550 mμ are replaced by a single peak at 528 mμ (Margoliash and Frohwirt, 1959). It is evident, therefore, that if cytochrome *c* is the chromophore, it is in the reduced state. The maxima and minima of the action spectrum fit quite well with those of the absorption spectrum. It will be seen, however, that although the heights of the action spectrum maxima are of the same relative order (415 mμ > 550 mμ > 520 mμ) as those of the absorption spectrum, the relative heights at 550 mμ and 520 mμ are different for the two spectra. While the action spectrum for spawning closely matches the reduced cytochrome *c* absorp-

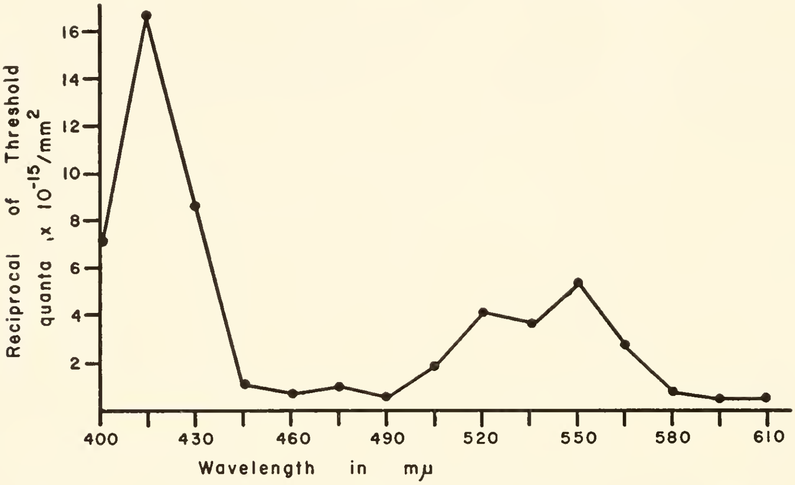

FIGURE 1. Action spectrum for light-induced spawning of *Ciona intestinalis.*

tion spectrum, the resolution attained by our system is not sufficient to do more than suggest that cytochrome *c*, or some other hemoprotein, may be the receptor material.

The role of hemoproteins in photobiological processes has been extensively investigated by Arvanitaki and Chalazonitis (1949, 1960, 1961). These workers, studying the effect of monochromatic light on the visceral ganglion of the gastropod *Aplysia*, have demonstrated that two chromophores are involved in light reception as measured by the electrical activity of isolated neurons. These pigments seem to act in antagonistic ways upon absorption of light. One pigment, a carotene-protein, generally produces a hyperpolarization of the membrane potential and inhibition of spiking. The other pigment, a heme-protein, produces a membrane depolarization and the initiation of spiking. The pigments are contained in granules just below the plasma membrane of the nerve cells, imparting a reddish hue to the cells. It is hypothesized (Chalazonitis, 1964) that the heme-protein, upon absorbing light, may pass an electron to the carotene-protein, thereby acting as a photoconductor. This transfer of electrons within the membrane is then visualized as opening channels for ionic flow. Thus a generator current is initiated which, if of sufficient intensity, may initiate action potentials. It is tempting to suggest that light absorbed by heme-proteins in *Ciona* may trigger a similar chain of events leading eventually to spawning. This, of course, implies absorption of light and action at the neuronal level. While it is true that the neural ganglion and numerous nerves of *Ciona* were illuminated in these experiments, other pigmented structures such as the tip of the gonopore and the neural gland, also received light. Studies are now under way to attempt a localization of the light absorbers and to investigate the neurophysiology of this response. Since the visceral ganglion is found deeply buried in the viscera of the intact *Aplysia*, it is extremely unlikely that light

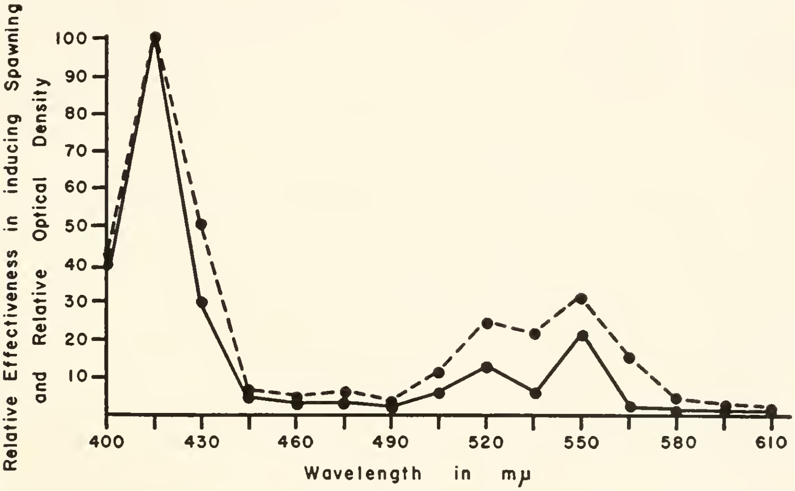

FIGURE 2. A comparison of the absorption spectrum of cytochrome *c* (solid line) with the action spectrum for spawning of *Ciona intestinalis* (dashed line).

can reach it to cause a behavioral response in such an animal. It is possible that the work reported here on the action spectrum for spawning of *Ciona* is the first demonstration of a hemoprotein involvement in a photo-induced behavioral response by any animal.

SUMMARY

1. The spawning of *Ciona intestinalis* with respect to light was studied, using both white light and monochromatic light.

2. A one-hour dark-adaption period followed by exposure to light resulted in spawning by 66.6% of the 884 animals tested.

3. Spawning occurs an average of 27.3 minutes after the onset of illumination.

4. Illumination need not be continuous until spawning occurs; the animals spawn when returned to the dark after a short illumination period, provided they have received enough energy.

5. The action spectrum for spawning suggests cytochrome *c* as a chromophore.

LITERATURE CITED

Arvanitaki, A., and N. Chalazonitis, 1949. Réactions bioélectriques neuroniques à la photoactivation spécifique d'une héme-protéine et d'une carotène-protéine. *Arch. Sci. Physiol.*, **3**: 27–44.

Arvanitaki, A., and N. Chalazonitis, 1960. Photopotentiels d'excitation et d'inhibition de defférents somata identifiables (*Aplysia*) activations monochromatiques. *Bull. Inst. Oceanog. (Monaco)*, **57**: No. 1164, 83 pp.

Arvanitaki, A., and N. Chalazonitis, 1961. Excitatory and inhibitory processes initiated by light and infra-red radiations in single identifiable nerve cells. *In:* Nervous inhibition: Proceedings of the Second Friday Harbor Symposium, Ernst Florey, Ed., Pergamon Press, New York, pp. 194–231.

Berrill, N. J., 1947. The development and growth of *Ciona intestinalis*. *J. Mar. Biol. Assoc.*, **26**: 616–625.

Carlisle, D. B., 1951. On the hormonal control of the release of gametes in ascidians. *J. Exp. Zool.*, **28**: 463–472.

Castle, W. E., 1896. The early embryology of *Ciona intestinalis* Flemming (L.). *Bull. Mus. Comp. Zool., Harvard*, **27**: 201–280.

Chalazonitis, N., 1964. Light energy conversion in neuronal membranes. *Photochem. Photobiol.*, **3**: 539–559.

Conklin, E. G., 1905. The organization and cell lineage of the ascidian egg. *J. Acad. Nat. Sci., Philadelphia*, **13**: 1–119.

Costello, D. P., et al., 1957. Methods for Obtaining and Handling Marine Eggs and Embryos. Mar. Biol. Lab., Woods Hole, Mass.

Hŭus, J., 1939. The effect of light on the spawning in ascidians. *Avhandlinger utgitt av Det Norske Videnskaps-Akademi I Oslo I. Mat.-Naturv. Klasse, No.* **4**: 5–49.

Hŭus, J., 1941a. Effects of physical factors on the spawning in ascidians. Temperature limits for spawning. *Avhandlinger utgitt av Det Norske Videnskaps-Akademi I Oslo I. Mat.-Naturv. Klasse, No.* **8**: 1–13.

Hŭus, J., 1941b. Effects of physical factors on the spawning in ascidians. Temperature and latent period. *Avhandlinger utgitt av Det Norske Videnskaps-Akademi I Oslo I. Mat.-Naturv. Klasse, No.* **9**: 2–12.

Millar, R. H., 1953. *Ciona.* L. M. B. C. Memoir XXXV. University Press, Liverpool.

Margoliash, E., and N. Frohwirt, 1959. Spectrum of horse heart cytochrome *c*. *Biochem. J.*, **71**: 571–572.

Rose, S. Meryl, 1939. Embryonic induction in ascidia. *Biol. Bull.*, **77**: 216–232.

GENETIC AND DEVELOPMENTAL STUDIES ON BOTRYLLUS SCHLOSSERI[1]

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The colorful compound ascidian, *Botryllus schlosseri*, has great promise for investigation in several important theaters of genetics, notably development and natural variation. In order for its potential to be realized as an experimental animal in these areas, a variety of preliminary studies have been undertaken. The results of these studies are reported here.

Botryllus has been studied extensively by Bancroft (1903), Berrill (1941a, 1941b, 1951, 1961), Oka and Watanabe (1957, 1959, 1960), and Sabbadin (1958, 1959, 1960, 1962, 1964), as well as by Scott (1934), Watterson (1945), and others. After Bancroft's early work on its natural history, development, species structure, and its property of colony fusion, Berrill used *Botryllus* as one major object of study in his broad and highly important series of contributions on development. More recently, Oka and Watanabe (on *Botryllus primigenus* and *Botrylloides*) and Sabbadin have addressed themselves to additional developmental problems, as well as to the genetic analysis of pigmentation and compatibility.

Although *Botryllus* is well described in the literature (Berrill, 1950; Van Name, 1945) and is exceedingly common just below the low water mark on pilings, eel grass, and under rocks, particularly in harbors, it is not a familiar organism even to many marine biologists, and a brief description is therefore in order. *Botryllus* colonies are of irregular shape and may be well over a foot in diameter, though usually much smaller. Each colony (Fig. 1) is composed of rosette-like *systems* of generally 5–18 *zooids*, each of which is like a solitary ascidian in form. The zooids, together with a vascular system which pervades them and the areas between and around systems, and which consists of blood vessels and ampullae (Fig. 2), are embedded in a gelatinous matrix which is maintained in a dynamic state by the activities of numerous amoeboid cells. The zooids' long axes are radially arranged in the systems. Their oral (incurrent) siphons are peripheral and open directly to the water; their atrial siphons open into the system's common atrial chamber which in turn communicates with the outside *via* a common atrial opening. The concentration of hydraulic force thus permits the powerful ejection of fecal pellets (and sperm); accordingly the system may be thought of primarily as a unit of egestion.

The oozoid resulting from the metamorphosis of a tadpole-type larva initiates the asexual formation of a colony by budding. Throughout the life of the colony, budding is synchronous, and when the buds become functional zooids, the previous

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[1] This was was supported by Research Grant GM 07810 of the National Institute of General Medical Sciences, United States Public Health Service.

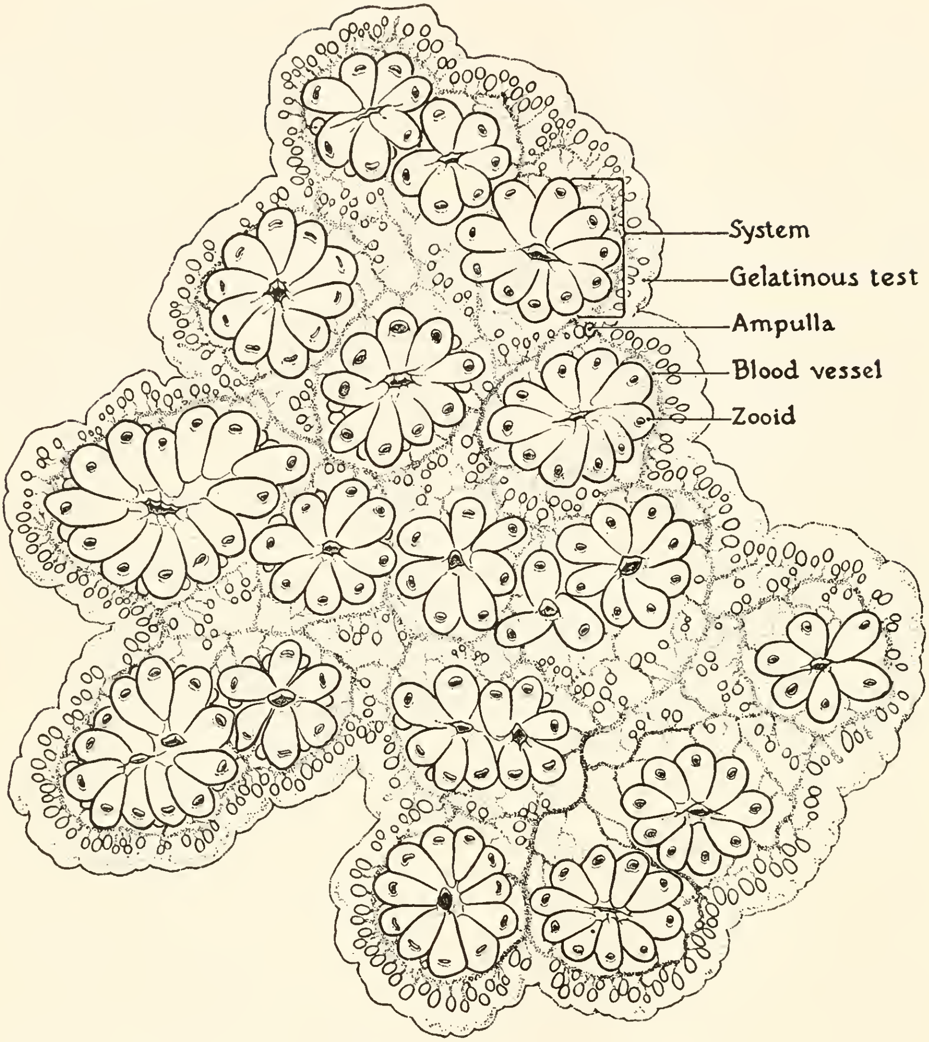

Figure 1. Habit sketch of *B. schlosseri* on glass.

generation of zooids is resorbed. Beginning with the oozooid, zooid size and pigmentation increase in each of the first five or ten asexual generations. During this period, first functional sperm and finally mature eggs make their appearance. The buds are produced at specific sites on the atrial wall, one per zooid at first, and later up to four. The dependence of functional gonads upon a certain zooid size suggested, and specific surgical experiments (Berrill, 1961) confirmed, that the degree of differentiation is dependent on mass in a manner reminiscent of the findings of Lopaschov (1935) and Grobstein and Zwilling (1953) in frog, chick, and mouse. Colonies under suboptimal conditions may mark time or even regress while the sequence of budding and resorption continues.

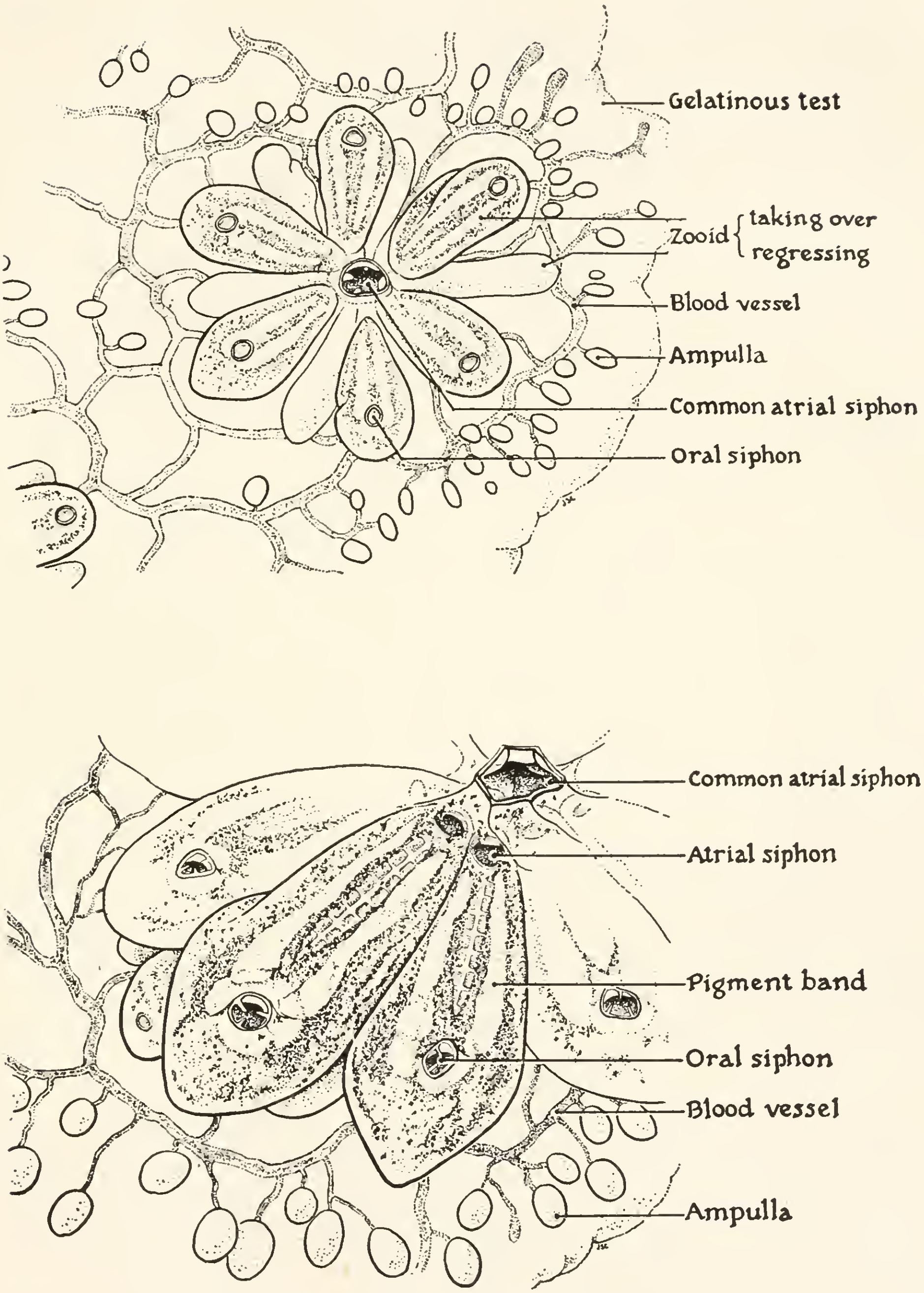
Gelatinous test
Zooid { taking over
regressing
Blood vessel
Ampulla
Common atrial siphon
Oral siphon
Common atrial siphon
Atrial siphon
Pigment band
Oral siphon
Blood vessel
Ampulla

FIGURE 2. Details of colony in Figure 1.

Sabbadin (1958) has also caused right-left inversion of asymmetry by disturbing early buds. This *situs inversus* is perpetuated faithfully in further atrially budded generations. We have repeated these observations.

Since all the zooids in a colony are ordinarily derived by budding from a single progenitor, the colony is a clone, and all the individuals share a color pattern which is distinguished easily from most of the myriad color patterns of surrounding colonies. Age and environmental influences on color patterns exist (Watterson, 1945) but are subject to independent analysis. Contiguous colonies are delineated by a clear discontinuity, generally bordered by tiers of vascular ampullae. Since the colonies occasionally fuse (the possibility apparently being based on genetically controlled affinities), mosaics do arise. These mosaics may become quite complex, since each generation of zooids is resorbed when its buds mature, and the systems can be rearranged radically—indeed, shuffled—as the number of zooids changes. The basis of the color pattern lies in the number and distribution of three kinds of pigment cell: blue (granular), white (granular—purine derivative), and orange (carotenoid in solution) (Sabbadin, 1959).

Experiments by Sabbadin (1959, 1962, 1964) show that certain components of color patterns are inherited in a simple Mendelian way. The availability of a large number of potential markers, together with other useful properties, suggests the feasibility of extensive genetic studies on *Botryllus*. This accessibility to classic genetic analysis is fortunate in view of the major modern problems for whose investigations it appears remarkably well suited. Sabbadin (1959) has pointed out that the tremendous variety of color patterns, once resolved into the activities of individual genes, would offer a way to study natural genetic variation by direct observation. In addition, of course, subsequent studies of the individuals observed and their progeny would add further substance to such an investigation. The primary concern of the present paper, however, is development. The remarkable powers of regeneration shown by *Botryllus* put it in the same league as many plants, such as carrot and tobacco; moreover, its structural complexity and the similarities of its larval development to that in some vertebrates add to its desirability for study. Finally, it is the hope of many animal geneticists to establish cell culture operations by which they can alternately treat cells as micro-organisms for mutational and recombinational studies, and grow them into adult organisms for detailed study of form and function. Thus an important part of the genetics of development may be accessible to analysis in *Botryllus*. The experiments to be described may be viewed as steps in this direction.

Laboratory Culture

Botryllus can easily be maintained in laboratory culture, provided that certain conditions are met. Cultures must be flat, rather than in clumps. The flat growth habit is automatic when larvae settle on glass slides or similar flat objects and metamorphose. Flat colonies from large mussel shells, boards, or similar natural surfaces can easily be removed and allowed to attach to glass; small clumps, if attached to a flat surface, will also spread out by the movement (on the order of several mm./day) of the existing systems and with subsequent growth.

The physical circumstances of the colonies in culture are critical. Beakers of still sea water serve well. Larvae will attach to glass slides, which can then be placed vertically, or even better, aslant with the *Botryllus* facing down. Horizontal mounting, with the colonies upside down, is best of all: the fecal pellets drop away, and contaminating filamentous algae are less likely to take hold. Saran wrap, to which the larvae readily attach, can be floated on culture medium also. For examination, it is inverted and submerged; it can be refloated when desirable. Also, for fusion compatibility tests, the Saran can be cut and colonies approximated, with a firm supporting substratum if desired. Zooids on the bottom of a vessel simply do not do very well.

Turbulence results in the presence of fecal pellets and other comparably sized detritus throughout the sea water: contact of such particles with the oral siphon or branchial basket causes reflex cessation of pumping and reversal of water flow by contraction of the body wall and thus prevents feeding and a normal flow of water through the individuals. If such a situation persists, the colony degenerates. Accordingly, aeration and stirring, if employed, require careful design.

Botryllus is tolerant of salinity changes. Concentration or dilution of sea water by 20% produces no ill effects, and short exposures to more extreme conditions (including distilled water) can be survived. The use of Instant Ocean, an artificial sea water, is advantageous from several points of view. It contains no organic substances and no predators or competitors, and it is a great convenience inland. Under conditions where evaporation is controlled or compensated and microorganisms do not multiply explosively, weekly changes of water suffice.

Cultures grow well between 18° and 28° C. Since water temperatures survived over the winter are much lower, it is likely that lower culture temperatures could be used, but growth would be very slow at best. It is also probable that even higher temperatures could be used, particularly where other conditions are optimal.

Cultures can sometimes survive for months without added food. The budding cycle proceeds slowly with a gradual reduction in size and number of individual zooids. This suggests that rapid regression and death are not due to starvation, and thus that a major problem in laboratory culture is the control of other organisms. Colony growth, of course, requires the addition of food (algae), either *via* running sea water or from algal cultures. *Cyclotella nana*, a centric diatom, appears to be the best food organism used so far. In f/2 medium (Guillard and Ryther, 1962) made with Instant Ocean the algal cultures reach concentrations of 1×10^6 cells/ml.; in f/2 medium made from sea water, 2×10^6 cells/ml. *Botryllus* colonies grow well in concentrations of $0.5–2.5 \times 10^5$ cells/ml. Indeed, young zooids under these conditions have on occasion developed four buds each, one more than the three considered maximal till now (Berrill, 1961), and the four buds have all become functional zooids in some cases.

Satisfactory feeding of any filter-feeder requires that two conditions be met: first, there must be enough food, and second, the concentration of this food must be high enough for an adequate feeding rate but not high enough to be harmful. In the present case, a concentration of $1–2 \times 10^5$ cells/ml. is used. This is a safe distance from the level at which the feeding system becomes clogged, too many algae accumulate on the dorsal lamina, periodic regurgitation takes place, and death eventually ensues. *Botryllus* is apparently not successful at intermittent feeding in constant high concentrations of food. A concentration of 5×10^5 cells/ml., for example, is accompanied by slow growth and poor appearance of the colonies.

Higher concentrations generally cause regression after a day or so. On the other hand, 0.5×10^5 (a concentration which also supports efficient feeding) has the disadvantage of providing only $\frac{1}{4}$ as many diatoms as 2×10^5 in a given vessel, thus necessitating a volume four times as great.

With just a few newly metamorphosed oozoids, culture vessel volume is no problem; but with colony growth, the removal of algae from the medium becomes rapid. On the basis of the time taken for a given colony to clear its water, I estimate that each zooid can easily filter 2×10^6 algae per day. (Not all of these are absorbed, as examination of the fecal pellets shows, but they are no longer available.) Thus, if a suspension of 2×10^5 algae/ml. is provided each day, the minimum culture vessel volume is about 10 ml. for each zooid. Since food intake becomes slower as the concentration falls, doubling this volume would be even better. A suspension of 1×10^5 algae/ml., allowing 40 ml./zooid, is probably optimal.

Closed vessels (or vessels covered with glass plates, Parafilm, *etc.*) are obviously convenient, particularly since stirring is not required. Polyethylene, through which oxygen and carbon dioxide can pass and water vapor cannot, suggests itself as a good cover (Walters and Williams, 1966). Constant light is acceptable and permits the diatoms to photosynthesize and thus produce oxygen. It also favors the growth of all algae, however, and may do more harm than good if certain filamentous forms are present. Budding and gonad development proceed similarly on all light regimes; only larval release (Costello *et al.,* 1957) seems to be influenced by light. In any event, large colonies require impractical volumes in standing culture, so continuous flow systems maintaining the concentration of algae within the desired range are preferable for them. The development of a recirculating aquarium for filter-feeders would be useful: the problem is merely one of finding an appropriate water filter.

For genetic studies, rapid growth and sexual maturation are desirable. Under the culture conditions described, performance is satisfactory. In the progeny of one mating, the colonies had from 5–22 zooids 22 days after metamorphosis (26 days after fertilization). Mature eggs are produced by cultured colonies less than 1½ months after metamorphosis; and once eggs are produced, of course, a new batch appears every 5–7 days, as long as conditions remain good.

Sabbadin (1960) has reported using *Chlamydomonas* (marine members of this genus are now called *Dunaliella*) and *Nitzschia* (perhaps what is now called *Phaeodactylum*) to feed isolated colonies at Chioggia, on the Lagoon of Venice. I am not convinced that I have given this combination an adequate test, but I have not been successful with it.

Predators, such as the snail *Mitrella lunata* and probably some flatworms and nematodes; competitors, such as filamentous algae, sponges, encrusting ectoprocts, *Bugula,* and entoprocts; and bacteria (whose activities may be competitive or direct) can all destroy cultures. In running sea water, *Mitrella,* sponges, and ectoprocts become an increasing problem as the summer progresses. In isolated culture, bacteria, algae, and entoprocts have proven more bothersome. Amphipods, harpacticoid copepods, and a variety of ciliates seem to coexist peacefully in *Botryllus* cultures as they do in the miniature jungles of wild colonies. But clean colonies derived from washed unhatched larvae do best.

Although in the long run *Botryllus* cultures require the maintenance of favorable conditions, they respond well to occasional rough treatment. For example, small colonies on glass have survived exposure to air for ten minutes or longer and microscopic examination under a coverslip for similar periods. Colonies accustomed to 25° C. have survived a day in the refrigerator, but not much longer.

THE REPRODUCTIVE CYCLE

It is of particular importance when dealing with an organism capable of selfing to have control of fertilization. Such control is achieved in *B. schlosseri* by fertilizing isolated eggs with isolated sperm (Milkman and Borgmann, 1963). It is believed that this is the first time external fertilization has been accomplished with a compound ascidian, and it depends upon removing the eggs at the right time. This in turn depends upon a detailed understanding of the timing of egg maturation and sperm maturation in relation to one another and to the asexual cycle. The present investigation has clarified these time relationships.

TABLE I

Timetable for one asexual generation

Day	Adult	Embryo	Testes	Bud	Egg	Testes
0	Takes over	Fertilized	0–1	Small	Small	
1	Darkens	Raspberry	1–2	Grows and		
2	Grows very	Tailbud	2–3	projects out		
3	little	Wraparound	2–4	between zooids		
4		Larva	3–5			pre-0
5		Released	4-remnants	Swells and	Full-	pre-0-0
6	Resorbed			takes over	sized*	0–1

* Enters atrial cavity, germinal vesicle breaks downs, egg fertilized.

Eggs develop in special chambers beside growing buds. They reach maturity when the buds replace the previous zooid generation. Since this is a fairly synchronous process (distant systems in a large colony may be several hours apart), one can obtain hundreds, even thousands, of eggs from a good-sized colony. As the new generation takes over, the eggs are pushed out of their chambers into the atrial cavity of the swelling bud. The germinal vesicle is in clear evidence in the eggs. During the next two to three hours, the following things happen in parallel: (1) the old zooids shrink down and no longer contain (or release) sperm; (2) the new zooids swell further and open their siphons; (3) the germinal vesicle breaks down and the eggs soon become fertilizable.

It should be added that the new zooid's testes generally do not release mature sperm until two days later. From this array of events, then, it follows that eggs will not be fertilized by sperm from the same colony–clone unless sperm are not forthcoming from elsewhere for two days, or unless the colony is so large that the first eggs become accessible and fertilizable before the last old zooids, often virile to the end, lose their mature sperm.

At the time of takeover, the testes contain very few mature sperm. That these are not released is indicated by the failure of newly mature eggs in the same colony or another to be fertilized by them. The proportion of mature sperm in a testis rises with time. The rate of release must be low at first; the testes ultimately reach a state of great fragility in which they contain nothing cellular except sperm oriented in parallel; at this point the sperm output of the colony must be several orders of magnitude greater than at first. Table I shows the timetable of sexual and asexual events in an adult colony. The budding cycle takes 5–7 days in the laboratory. Illustrated in Table I is a 6-day cycle. The embryonic stages' designations used here for convenience, reflect their appearance. The raspberry stage is a gastrula; the wraparound is still rather opaque and spherical, with the tail wrapped around. Later the embryo clears and elongates into the larva, whose subsequent release appears to be influenced by its light regime (Costello *et al.,* 1957). The buds are not visible until just before takeover, except in very flat colonies. Otherwise they are concealed in the interior of the colony mass.

TABLE II

Distribution of testis stages vs. embryo stage in individual colonies

Colony	Embryo stage	Testes in each stage (N)					
		0	1	2	3	4	5
1	Raspberry	0	1	22	2	0	0
2	Raspberry	0	4	23	3	0	0
3	Tailbud	0	2	22	1	0	0
4	Tailbud	0	0	9	11	1	0
5	Early wraparound	0	1	13	2	0	0
6	Wraparound	0	0	1	22	0	0
7	Larva	2*	1*	1	18	0	0
8	Larva	0	0	0	3	17	1
9	Tadpole	1*	1*	0	0	0	15
10	Tadpole rare	0	2	0	0	0	20

* Possibly taken accidentally from buds (see text).

Table II shows the degree of uniformity among the testes in a colony. Colonies were staged according to their embryos, whose stages are very uniform indeed under normal circumstances. Since these colonies were taken from a dock crowded with *Botryllus,* their eggs were surely fertilized at the earliest possible moment. Now within a given colony, the testes appear to be fairly synchronized, though there is some scatter. (Exceptionally immature testes in an otherwise mature group may have been taken accidentally from a bud.) On the other hand, a comparison from colony to colony suggests that the phase relationship is not constant for the species, though of course its range of variation is not great. This variability among colonies must be kept in mind, for it, too, affects the possibility and time of selfing.

It should be clear from this description that there is no sudden onset of paternal competence in a *B. schlosseri* colony. Mature sperm are seen well before they are normally released; crushed testes achieve a small percentage of fertilization at early stages also. Table III illustrates the quantitative nature of testis maturity, comparing testis stage with per cent fertilization. Eggs from the same batch were placed with crushed testes of various stages and cleavage was observed.

Conjectures involving storage of sperm or other complex mechanisms of fertilization can be discarded because eggs can be taken at the right moment and fertilized. Similarly, there is no evidence of egg-sperm incompatibility. The only technical difficulty is that eggs isolated with germinal vesicles intact will never be fertilized; and it is probable that they do not mature until about an hour after breakdown. Subsequently the eggs can be removed and fertilized. The actual removal consists of slitting the zooids and gently pressing out the eggs: this is a very easy procedure, and the zooids repair the damage within 24 hours.

Large wild colonies containing many eggs per zooid (I have removed as many as 11 from one) can be staged and isolated about a day before takeover. As Sabbadin points out (1959), the property of fusion places the clonal nature of any wild colony in doubt, however, and it is certainly better to raise breeding colonies from tadpoles. At any rate, for eggs each colony should be sequestered before the new siphons open. For large colonies, a lucite container, divided into radial sectors, has been used to isolate up to ten potential egg sources. This device, built to operate like a reverse *Botryllus,* distributes water from a common central tube; outflow is peripheral. Where running sea water is not available, large chambers containing no food can be used. Here stirring involves no risk. A closed vessel, together with the great efficiency of the *Botryllus* pumping system, raises the problem of selfing once more (which is minimized by the constant washing of continuous flow). Accordingly, egg sources should be washed and isolated shortly before the new siphons open.

TABLE III

Fertilization efficiency vs. testis stage

Testis stage	Eggs (N)	% Fertilized
1	55	0
3	135	15
3½	80	28
3½	62	29
4+	83	46

Eggs to be fertilized *in vitro* are placed in a Syracuse dish of (natural or artificial) sea water. Testes are then added. After the eggs are swirled to the center of the dish, the testes are crushed, and the eggs are nested in a thick layer of sperm like berries in whipped cream. Polyspermy is fortunately not a problem, and fantastic quantities of sperm are required in comparison, for example, to the amounts needed to fertilize sea urchin eggs. This situation, seen somewhat less spectacularly in other tunicates (Costello *et al.,* 1957), suggests that imperfections still remain in the *in vitro* method, even though 100% fertilization can be achieved.

DEVELOPMENT OF FERTILIZED EGGS

Eggs fertilized *in vitro,* as well as early embryos removed from zooids, can develop into mature larvae and subsequently metamorphose. Until recently, techniques for permitting such *in vitro* development were complicated and unreliable at best; this was a major obstacle to the use of controlled mating. Now, however, the simple expedient of placing the early embryos on a piece of filter paper in a vessel containing Instant Ocean provides a good method of raising them. The filter paper apparently serves two purposes: it provides for some circulation even where the egg rests on its surface (Saran doesn't work as well), and it is not as hard as glass. Eggs resting directly on glass become deformed and bacteria accumulate at the point of contact and almost invariably attack and destroy the embryo by the time of the gastrula stage or thereabouts. Filtered sea water is also satisfactory. If evaporation is prevented, the water need not be changed, but a change seems to result in healthier larvae. Hundreds can be raised in a finger bowl. Before eclosion, the larvae are transferred to an appropriate vessel containing slides or Saran for attachment, since the time of eclosion and that of attachment and metamorphosis are variable and hard to control.

The Vascular System and Vascular Budding

The vessels which pervade the zooids and outlying areas also connect to the many vascular ampullae which are found in tiers at the periphery of the colony, in rings around each system, and irregularly scattered throughout the matrix between systems. Differential interference (Zeiss-Nomarski system) microscopy, which permits undistorted high-magnification observation of optical sections of relatively thick preparations, shows that the vessels and ampullae are very delicate structures. Their walls are essentially one cell thick. Ordinary light microscopy of young colonies under coverslips permits similar observations. The blood circulating through the systems is moved, not only by the hearts in the various zooids, but by contractions of the vascular ampullae. This is confirmed by motion pictures, again using the Nomarski microscope, which show localized contractions within each ampulla and thereby eliminate passive elastic contraction as the basis of their periodic reductions in size. Moreover, removal of all the zooids and buds does not stop circulation in the remaining outlying vessels. Circulation continues for hours, and longer.

Isolated regions of the vascular system are of great interest because they can regenerate entire zooids and, ultimately, whole colonies, in spite of their simple structure and composition. Oka and Watanabe (1957, 1959) demonstrated vascular budding in *B. primigenus* and in *Botrylloides;* Watkins (1958) suspected it in *B. schlosseri;* and Byrne and I demonstrated it (Milkman and Byrne, 1961). In *B. schlosseri* vascular budding has been seen only when all the zooids are removed, but the possibility remains that under conditions of very rapid growth it occurs in intact colonies also. During the first few days following excision of the zooids (and buds!), the ampullae consolidate into one or a few rather highly pigmented masses, and these structures may now gradually begin to resemble miniature zooids, whose size is that of zooids newly metamorphosed from larvae. Histological study of this process has not yet been made in *B. schlosseri,* but even at the gross level there appear to be differences between vascular budding in this species and those reported by Oka and Watanabe. In less than a week, the tiny functional zooids are feeding and growing.

This remarkable regenerative ability leads one to wonder if *B. schlosseri* cells can be cultured and then be induced to form zooids in a manner analogous to vascular budding. It was considered useful first to make further inquiry into the nature of the cells determining the characteristics of the zooids so produced. To this end, advantage was taken of the ability of morphologically different colonies to fuse (Bancroft, 1903). Colonies differing in color pattern were made contiguous; a small proportion of them fused gelatinous tests and vascular systems, thus permitting (indeed, necessitating) a complete interchange of blood cells. A week after fusion, all zooids and buds were removed from the fused colonies, leaving only their common vascular systems (and tests). Separate portions of each colony were taken before fusion and maintained for comparison with the zooids to be regenerated. In over 30 cases, the zooids produced by vascular budding in turn produced systems identical to the parent systems originally present at the site of the bud. There were no exceptions. This proves that, whatever the contributions of the freely circulating blood cells, the fixed cells of the delicate vascular walls (or conceivably of the test) determine the phenotype of the regenerated zooids (Milkman and Therrien, 1965). Figure 3 illustrates the experiment. No buds were seen at the original fusion border; this is probably a statistical matter. Perhaps a somatic recombinant could be obtained if a bud of dual origin occurred.

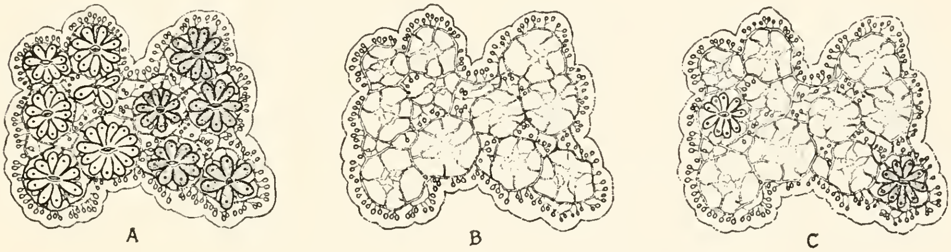

Figure 3. Experiment on vascular budding in combination of two fused colonies. Note similarity of system derived from vascular buds to systems derived from vascular buds to systems originally present at the same site. See text for further details.

In these experiments, it was necessary for the systems produced to reach a steady-state with respect to pigment cell concentration. During the first several atrial budding generations after the vascular bud is formed, the proportion of pigment cells is unusually high, as it is in the original coalesced vascular tissue. Gradually this proportion decreases and is maintained at a steady level corresponding to that in the parent. This situation is reminiscent of the changes in pigmentation in the first few bud generations coming from an oozoid; but in the latter case, pigment cell concentration starts low and then rises to a maintained level (Bancroft, 1903; Watterson, 1945; Sabbadin, 1959). Moreover, when the old zooids are resorbed, their pigment cells assemble in the ampullae and are gradually released and taken up by the new generation of zooids. Thus buds begin by being rather pale; their sudden expansion at takeover time spreads out their relatively few pigment cells and makes them paler; and they gradually darken the next day. From these observations, old and new, it can be seen that the distinctive pigmentation of a mature *Botryllus* colony depends basically on its own particular genotype, and that it is affected more immediately during the ontogeny of the colony by the relative rates of formation of pigment cells and their concentration in a given stage (oozoid or regenerating tissue).

The obvious next step in this area of investigation, which has not yet been undertaken, is an attempt at culturing vascular tissue, with or without blood cells. It is of interest that Freeman (1964) has found in *Perophora* that only lymphocytes, of the several varieties of blood cells, are necessary for budding, which occurs normally in that form at intervals at the growing end of the stolon, which carries bloods among the zooids. Since he arrested all cell division by irradiation and subsequently injected untreated cells of a given type, it is clear that these cells form the body of the newly budded zooids, unless (as seems unlikely) large numbers of non-dividing cells are mobilized from existing zooids once the blood cells initiate budding. Our findings do not rule out the participation of blood cells, but they suggest that the structure of the *Botryllus* zooid formed in vascular budding is controlled by the derivatives of the vascular tissue, or conceivably (though the appearance of the bud lends no support to this alternative) cells in the test. The quantitative changes in pigmentation suggest that different regions in the zooids have different affinities for each kind of pigment cell and that a mass action relationship determines the disposition of pigment cells at any time. The mobility of pigment cells that has been observed supports the view that, though they may lodge in a particular place for a considerable time, they are never permanently fixed.

TABLE IV

Results of representative crosses

Cross	Phenotypes	Genotypes	Phenotypes in offspring	
1 (self)	PB × PB	PP Bb × PP Bb	20 P:0 p	15 B:5 b
2	pB × PB	pp Bb* × PP BB	15 P:0 p	15 B:0 b
3	Pb × PB	P-† bb × P-† Bb*	19 P:0 p	7 B:12 b

Pigment band: P presence, p absence. Black ground color: B presence, b absence.
* Bb genotype indicated by another cross of same parent (not shown here).
† At least one should be PP.

GENETIC CROSSES

The crosses we have performed so far have been preliminary in nature and lead to three conclusions. First, selfing is general enough to suggest the absence of any important self-incompatibility system (except, of course, the highly effective difference in time of maturation of eggs and testes in a given colony). Second, Sabbadin's conclusion that the presence of a pigment band may be inherited in a simple, dominant Mendelian fashion is supported, although neither his data from individual crosses (Sabbadin, 1959, 1962, 1964) nor ours definitively exclude additional possibilities. Third, it is clear that the tremendous variety of offspring produced from a cross of any two colonies taken from nature defy extensive analysis: several generations of selfing are required to produce colonies sufficiently homozygous to be useful for the study of a large number of traits. Such a program of selfing necessitates laboratory culture methods capable of supporting sexual reproduction consistently; even now that we have such methods, any major degree of heterosis might delay or prevent the acquisition of homozygous colonies.

Table IV contains the results of some representative crosses. Putative parental genotypes for pigment bands are assigned tentatively; it is not really clear at this point that the inheritance of black ground color is simple. The numbers are quite small, but since these crosses were set up the techniques for getting good yields have been improved greatly.

There is one detrimental result of *in vivo* selfing of the usual type: when self-fertilization takes place two days after the normal time, the larvae are not ready for release at takeover time (see Table I). The colony seems unable to adjust its asexual schedule; the old zooids regress slowly and incompletely while they contain larvae. Concomitantly, the buds do not complete their last stages of development: they appear undersized and do not become contiguous with other zooids to form normal systems. Thus the colony dies, though many larvae escape. In exceptional cases of earlier selfing due to fertilization by remaining zooids of the previous generation or by unusually advanced testes of the current generation, this collapse may not occur. Actually, the fortunate expedient of refrigerating testes up to four days may be employed; sperm from these testes fertilize the eggs of the next generation perfectly. In addition, the possibility also exists of separating a colony into parts and staggering them at different temperatures.

Where traits are inherited in a simple Mendelian fashion, the alleles responsible can be followed in populations. As Sabbadin has suggested (1959), *Botryllus* is of particular interest because in animals two alleles associated with a striking phenotypic difference rarely both have high frequencies. The pigment band's presence appears to be dominant over its absence, although Sabbadin (1964) believes that multiple alleles account for some of the variants in pigment band form. At any rate, among 100 colonies on the M. B. L. Supply Dock in Woods Hole, 63 had pigment bands and 37 did not. If the conditions for the Hardy-Weinberg law obtained, the frequency of the "absence" allele was $\sqrt{0.37} = 0.6$, while that of the "presence" allele, or class of alleles, was $1 - 0.6 = 0.4$. Extension of these observations over space and time should be quite easy and may lead to useful conclusions about population sizes and related matters.

Chromosome Number

Colombera (1963), using either gallocyanin or gentian violet, together with preliminary aceto-orcein or aceto-carmine staining, on testes, buds, and cleaving eggs, has concluded that the haploid number of chromosomes in *B. schlosseri* is 16. Therrien and I (Milkman and Therrien, 1965) studied cleaving eggs using the Feulgen technique, blockading the cytoplasmic aldehyde groups with phenylhydrazine before hydrolysis. (Pronase had removed the chorion.) We estimated the haploid number to be 7 or 8. It is possible that this disparity has a real basis, or, of course, that our conclusions are incorrect. Colombera points out that 16 is rather high for ascidians, but that *Tethyum plicatum*, of the Styelidae (a family close to, or including, the botryllids) also has a haploid number of 16.

Handling of Blood

In my laboratory, Dr. Arnold Kahn has found it easy to remove and reinject *Botryllus* blood. As much as $\frac{1}{4}$ ml. of blood has been taken from a colony at one time, suggesting that the alternate passive elastic stretching and active contraction of ampullae are ordinarily responsible for the periodic reversal of peripheral blood flow. Blood cells and test cells survive in culture for up to three days but do not multiply. The ability to remove and inject blood easily and without injury permits one to attempt to confer fusion compatibility. It is also conceivable that intravenous feeding alone can support the growth of a Botryllus colony or isolated vascular system, and that nutritional studies at this level might lead to a wide variety of interesting findings.

General Discussion and Conclusions

The basic technical obstacles having been overcome, we can now look forward to extensive genetic studies on *Botryllus.* Inland culture techniques permit year-round propagation of strains, together with long-term crossing and selection programs. The ever-present risk of selfing, which cannot be controlled *in vivo,* is eliminated by the use of *in vivo* matings, which also permit multiple crosses involving one set of eggs or one set of testes. It may be concluded, then, that *Botryllus schlosseri* is ripe for teaching and experimental use. In anticipation of its increased popularity, in view of its appearance and habitat, and to remedy a current defect, the vernacular name "harbor stars" is now suggested.

This work has been done with the collaboration and assistance of Sylvia Byrne and Edward Therrien (NSF Undergraduate Research Participants), Martha Borgmann and Judith Pederson. Dr. Luigi Provasoli, Dr. Robert Guillard, and Mrs. Helen Stanley have been most generous with algal cultures, materials, and counsel. Dr. Martha Baylor suggested the fusion-vascular budding experiment. Dr. Robert D. Allen provided the Nomarski optics and made the movies. The illustrations are by Julia S. Child.

Summary

1. Properties of *Botryllus schlosseri* which give it outstanding promise for studies in developmental genetics are reviewed.

2. Laboratory culture procedures, *in vitro* fertilization, and a method for raising embryos *in vitro* are described. Controlled successions of complete life cycles can now be achieved in any laboratory.

3. Experiments involving colony fusion, subsequent vascular budding, and the analysis of color patterns in resultant systems suggest that cells of the simple vessel walls govern the morphology of the regenerated zooids.

4. Results of some preliminary genetic crosses are reported.

LITERATURE CITED

Bancroft, F. W., 1903. Variation and fusion in compound ascidians. *Proc. Calif. Acad. Sci., series 3,* **3**: 137–186.

Berrill, N. J., 1941a. The development of the bud in *Botryllus. Biol. Bull.,* **80**: 169–184.

Berrill, N. J., 1941b. Size and morphogenesis in the bud of *Botryllus. Biol. Bull.,* **80**: 185–193.

Berrill, N. J., 1950. The Tunicata. Ray Society, London.

Berrill, N. J., 1951. Regeneration and budding in tunicates. *Biol. Rev.,* **27**: 456–475.

Berrill, N. J., 1961. Growth, Development, and Pattern. Freeman, San Francisco.

COLOMBERA, D., 1963. I cromosomi di *Botryllus schlosseri* (Ascidiacea). *Ric. Sci.*, **33** (II-b). 443–448.

COSTELLO, D. P., M. E. DAVIDSON, A. EGGERS, M. H. FOX AND C. HENLEY, 1957. Methods for Obtaining and Handling Marine Eggs and Embryos. Marine Biological Laboratory, Woods Hole, Mass.

FREEMAN, G., 1964. The role of the blood cells in the process of asexual reproduction in the tunicate *Perophora viridis*. *J. Exp. Zool.*, **156**: 157–183.

GROBSTEIN, C., AND E. ZWILLING, 1953. Modification of growth and differentiation of chorio-allantoic grafts of chick blastoderm pieces after cultivation at a glass clot interface. *J. Exp. Zool.*, **122**: 259–284.

GUILLARD, R. R. L., AND J. H. RYTHER, 1962. Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt, and *Detonula confervacea* (Cleve) Gran. *Canad. J. Microbiol.*, **8**: 229–239.

LOPASCHOV, G., 1935. Die Entwicklungsleistungen des Gastrulamesoderms. *Biol. Zentralbl.*, **55**: 606–615.

MILKMAN, R., AND M. BORGMANN, 1963. External fertilization of *Botryllus schlosseri* eggs. *Biol. Bull.*, **125**: 383.

MILKMAN, R., AND S. BYRNE, 1961. Recent observations on *Botryllus schlosseri*. *Biol. Bull.*, **121**: 376.

MILKMAN, R., AND E. THERRIEN, 1965. Developmental and genetic studies on the compound ascidian, *Botryllus schlosseri*. *Biol. Bull.*, **129**: 417.

OKA, H., AND H. WATANABE, 1957. Vascular budding, a new type of budding in *Botryllus*. *Biol. Bull.*, **112**: 225–240.

OKA, H., AND H. WATANABE, 1959. Vascular budding in *Botrylloides*. *Biol. Bull.*, **117**: 340–346.

OKA, H., AND H. WATANABE, 1960. Problems of colony-specificity in compound ascidians. *Bull. Marine Biol. Sta. Asamushi*, **10**: 153–155.

SABADDIN, A., 1958. Analisi sperimentale dello sviluppo delle colonie di *Botryllus schlosseri*. *Arch Ital. Anat. Embriol.*, **63**: 178–221.

SABADDIN, A., 1959. Analisi genetica del policromatismo di *Botryllus schlosseri* (Pallas) Savigny (Ascidiacea). *Boll. Zool.*, **26**: 221–243. (Translation by Roger Milkman available on request.)

SABADDIN, A., 1960. Ulteriori notizie sull'allevamento e sulla biologia dei Botrilli in condizioni di laboratorio. *Arch. Oceanogr. Limnol.*, **12**: 97–107.

SABADDIN, A., 1962. Bande intersifonali di pigmento purinico in *Botryllus schlosseri* (Ascidiacea) e loro determinazione genetica. *Boll. Bool.*, **29L**: 721–726.

SABBADDIN, A., 1964. The pigments of *Botryllus schlosseri* and their genetic control. *Ric. Sci.*, **34**: (II-b) : 439–444.

SCOTT, F. M., 1934. Studies on the later embryonic development of Tunicata: *Botryllus schlosseri* and *Amaroecium constellatum*. Ph.D. thesis, Columbia University, New York.

VAN NAME, W. G., 1945. The North and South American ascidians. *Bull. Am. Mus. Nat. Hist.*, **84**: 1–476.

WALTERS, D. R., AND C. M. WILLIAMS, 1966. Reaggregation of insect cells as studied by a new method of tissue and organ culture. *Science*, **154**: 516–517.

WATKINS, M. J., 1958. Regeneration of buds in *Botryllus*. *Biol. Bull.*, **115**: 147–152.

WATTERSON, R. L., 1945. Asexual reproduction in the colonial tunicate, *Botryllus schlosseri* (Pallas) Savigny, with special reference to the developmental history of intersiphonal bands of pigment cells. *Biol. Bull.*, **88**: 71–103.

ENDOSKELETAL CARTILAGE IN A MARINE POLYCHAETE, EUDISTYLIA POLYMORPHA

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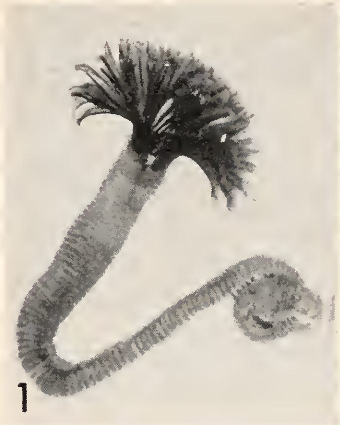
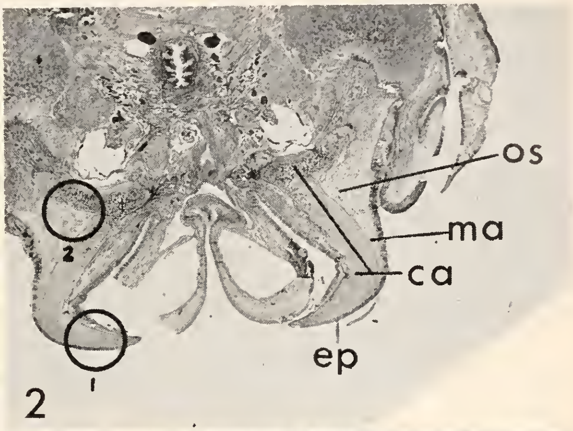
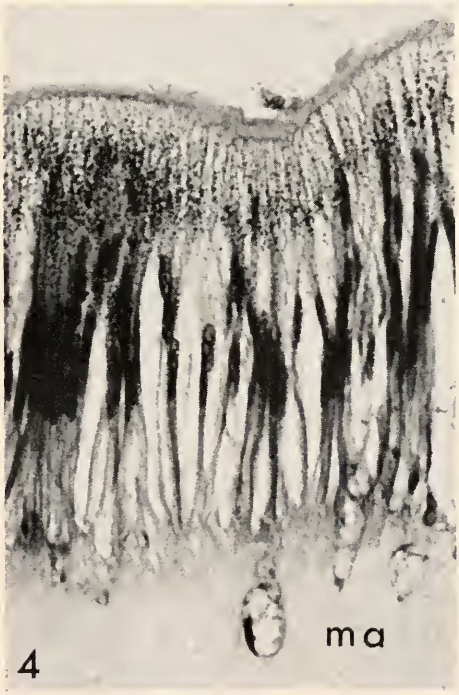
The nature of the endoskeletal tissues of certain marine worms has been the subject of controversy in the past. Claparède (1873) described such tissues as part of the tentacular and tentacle-supporting complexes in *Myxicola infundibulum* and *Spirographis spallanzani*, and considered them to be cartilage. Viallanes (1885), Nowikoff (1912) and Hempelmann (1928) described such tissues in the above and other polychaetes, and also considered them to be cartilage. However, in a brief report, Krukenberg (1882) said he could extract neither gelatin nor "mucin" from the tissues referred to above. He therefore concluded they could not be true cartilage, and that instead they were "cartilage-like." Subsequently, several other workers studied these endoskeletal tissues, and, basing their judgments primarily upon Krukenberg's negative findings, also decided that the tissues in question were not cartilage (Nicol, 1930; Evenkamp, 1931; Thomas, 1940).

The above problem is included in the broader question of the existence or nonexistence of cartilage tissues in invertebrates. Nowikoff (1912), on the basis of histologic, cytologic and also some chemical criteria, concluded that invertebrates did possess true endoskeletal cartilage tissues, while Schaffer (1913, 1930) argued that the invertebrate tissues in question were not cartilage. Schaffer (1930) considered that the invertebrate tissues in question were "chondroid," "chordoid" or "cartilage-like," and that "true" cartilage was found *only* in the vertebrates (1930, p. 210). In 1940, L. Hyman in her classic treatise on the invertebrates wrote that true cartilage was absent in invertebrates, which, she said, tend to secrete external noncellular rather than internal cell-containing hard parts (p. 281). Subsequently, Romer (1942) suggested that cartilage arose in the vertebrates as an embryonic adaptation to stresses and deformations produced by rapid growth. Since then, the view seems to have prevailed amongst most biologists that cartilage is a uniquely vertebrate tissue, as expressed more recently, for example, by Pritchard (1956) and Romer (1964). Indeed, most textbooks and monographs on vertebrate bone and cartilage, and most textbooks of invertebrate zoology, rarely mention invertebrate cartilage tissues. It should be noted, however, that a small number of individuals have continued to write of certain invertebrate cellular endoskeletal tissues as being cartilage (Person and Fine, 1957; Raven, 1958; Lash, 1959; Person and Philpott, 1963a).

In this paper, in further support of the contention that true endoskeletal cartilages are found in invertebrate animals, we present a beginning study of the histology and chemistry of cartilage tissues which form part of a rather complicated cellular endoskeletal complex in the marine polychaetous annelid, *Eudistylia polymorpha.*







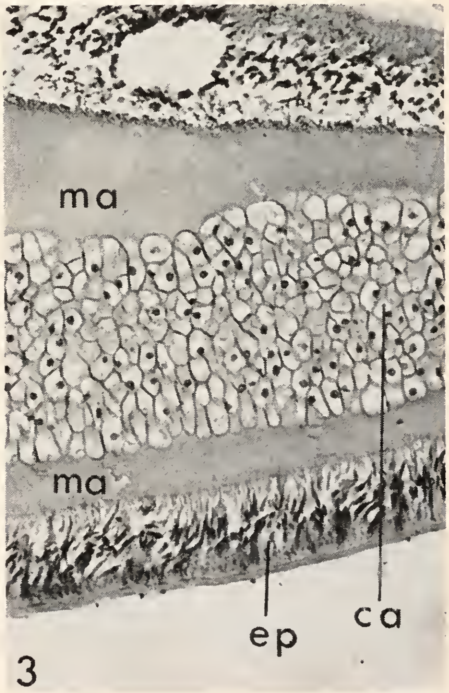
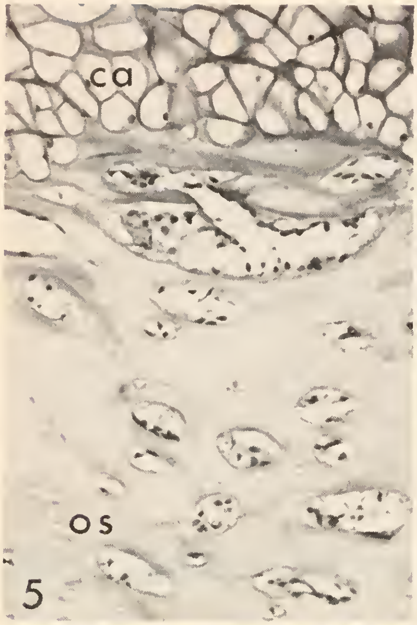


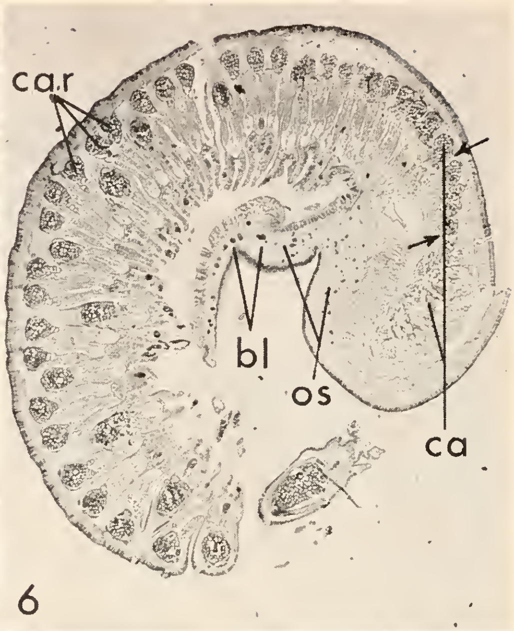
FIGURE 1. *Eudistylia polymorpha,* 8 inches long, removed from tube and photographed alive.

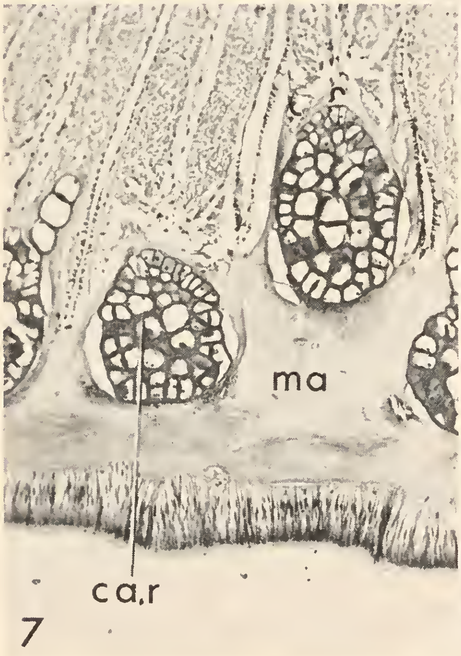
FIGURE 2. *Eudistylia polymorpha,* cross section of animal at level of base of crown: *ep* = epithelium, *ca* = cartilage, *ma* = matrix, *os* = osteoid-like tissue; magnification 18×.

FIGURE 3. *Eudistylia polymorphia,* higher-power view of circle I in Figure 2: *ep* = epithelium, *ca* = cartilage, *ma* = matrix; magnification 200×.

FIGURE 4. *Eudistylia polymorpha,* higher-power view of epithelial cells which appear to be secreting matrix (*ma*); magnification 500×.







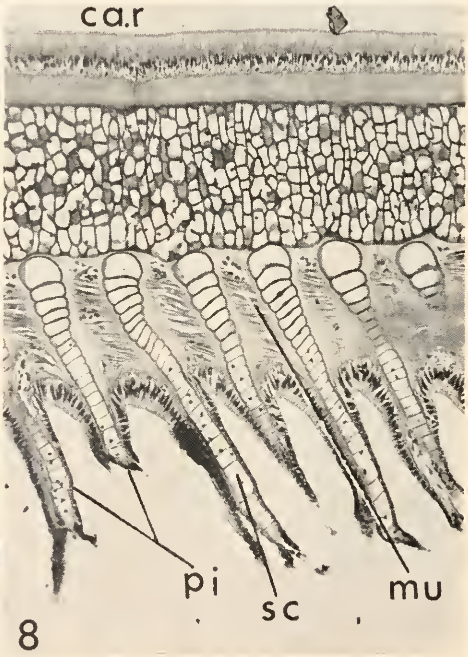


FIGURE 5. *Eudistylia polymorpha,* higher-power view of circle #2 in Figure 2: *os* = osteoid-like tissue, *ca* = cartilage; magnification 200×.

FIGURE 6. *Eudistylia polymorpha,* cross section through the base of a cluster of tentacles: *ca r* = cartilage rods, arrows (↑) show origin of cartilage rods from basilar mass of cartilage (*ca*), *os* = osteoid-like tissue, *bl* = blood; magnification 18×.

MATERIALS AND METHODS

Eudistylia polymorpha (Fig. 1) was collected and shipped *via* air express to the Marine Biological Laboratory, Woods Hole, Mass., by Dr. R. C. Fay, Pacific Biomarine Supply Co., Venice, California. The animals were active on arrival and were kept in their tubes in a sea tank for several days, following which they were sacrificed. For histologic study, tissues were placed in 10% neutral formalin and processed routinely to make hematoxylin-eosin stained sections. Fresh-frozen (non-fixed) sections were cut to approximately 10–15 μ and were stained with 0.01% toluidine blue at pH 3.5. For chemical analyses, the tissues were dissected and trimmed under a binocular microscope, and placed in cold acetone. For analysis of acid mucopolysaccharides, the tissues were digested with crystalline papain, and the acid mucopolysaccharides isolated and characterized chemically and physically by procedures described elsewhere (Mathews and Glagov, 1966). Hydroxyproline content was kindly determined by Dr. Milton Levy according to the method of Stegemann (1958).

OBSERVATIONS AND RESULTS

Gross observations

The endoskeletal tissues of *Eudistylia* are located anteriorly in the animal as supporting structures for the "feather-duster"-like tentacular complex which forms the crown of the animals. The main clusters of tentacles, of which there are two (see Fig. 1), are supported by a basal mass of cellular endoskeletal material, composed of cartilage and an "osteoid-like" tissue. (As used in this paper, the term "osteoid" refers to the vertebrate tissue identified histologically as the premineralized matrix in which apatite crystals are later formed during the process of bone formation.) These tissues are never mineralized. From this basal endoskeletal complex are also given off individual cartilage rods, each of which supplies a fairly rigid, but flexible, structural core for a single tentacle. From each rod, in turn strands or columns of cells are given off, to provide individual structural cores for the numerous villus-like pinnae, which project from each tentacle along its entire length. The pinnae exert a beating motion, creating water currents which sweep food down along the tentacular grooves, to facilitate ingestion by the mouth.

Histology

Basilar endoskeletal complex. In Figure 2, a low power cross section made at the level of the basilar endoskeletal complex is shown. In this section we will locate structures of interest, which will then be shown at higher magnifications. The outer surface of the animal is covered by a tall columnar epithelium (*ep*). In the region of the endoskeletal complex, the epithelium overlies and appears to secrete a relatively homogeneous eosinophilic matrix (*ma*). In some regions, this

FIGURE 7. *Eudistylia polymorpha,* higher-power view of cartilage rods (*ca r*), which support individual tentacles, and surrounding matrix (*ma*) which is continuous with osteoid-like tissue seen in Figures 2, 5 and 6; magnification 85×.

FIGURE 8. *Eudistylia polymorpha,* sagittal section of a tentacle and its pinnae (*pi*) : *ca r* = cartilage rod, *mu* = muscle bundles, *sc* = strands of cells which support pinnae; magnification 85×.

matrix is penetrated by an extensive vascular complex, giving to the tissue a bone-like or "osteoid-like" appearance (*os*) (it is stressed again that these tissues never mineralize). Adjacent to the matrix and "osteoid-like" tissue are masses of cartilage tissue (*ca*). In Figure 3, a higher power view of the circular area #1 (marked in Fig. 2) is shown. In this region, a relatively homogeneous character of the matrix (*ma*) is evident, as well as the abundantly cellular nature of the adjacent cartilage (*ca*). The cartilage matrix is comprised of thin seams of intercellular material. In Figure 4, the eosinophilic, granule-laden, tall, columnar epithelial cells are pictured at higher magnification. These cells bear a marked resemblance to ameloblasts or odontoblasts of vertebrate tissues, as well as to the odontoblasts of invertebrate radula-forming tissues in gastropod molluscs (Raven, 1958). Figure 5 shows a higher magnification of the circular area # 2 (marked in Fig. 2). Note the strong resemblance of the "osteoid-like" material (*os*) to vertebrate bone tissue in section. The cartilage in this region (*ca*) is quite cellular, but its intercellular matrix is thicker and more rigid than that seen in the cartilage in Figure 3.

TABLE I

Chemical analysis of acid mucopolysaccharide of Eudistylia cartilage compared to theoretical for chondroitin sulfate of mammalian cartilage, expressed as mole ratio to galactosamine

	Eudistylia	Theoretical
Galactosamine	1.00	1.00
Glucosamine	0.03	0.00
Uronic acid	1.07	1.00
Nitrogen	1.20	1.00
Sulfate	1.88	1.00

Tentacular complex. In Figure 6 is a cross-section of the worm made at a level slightly anterior to that from which the previous figures were made. The cartilage rods (*ca.r*) which provide internal support for each of the tentacles are strongly eosinophilic and stand out like vascular bundles seen in cross sections of plant tissues. The origin of the tentacular cartilage rods (*ca.r*) from the basilar cartilage masses (*ca*) is seen in the areas marked by the arrows (↑). One can also see at this level that the channels which penetrate the osteoid-like matrix (*os*) are part of the vascular system which contains a spirographis heme-containing blood pigment (*bl*) (Crescitelli, 1945). Figure 7 shows several cartilage rods at higher magnification (*ca.r*). A sagittal section of the portion of a tentacle, together with its associated pinnae, is seen in Figure 8. The central rod of cartilage (*ca.r*) gives off strands of cells(*s.c.*) which course to the very tips of the pinnae (*pi*). Note the beautiful, almost plant-like regularity in the cellular bio-architecture of these tissues. Note also that thin bundles of muscle (*mu*) run between each of the pinnae. This muscular system undoubtedly aids in the beating movements made by the feathery pinnae.

Toluidine-blue metachromasia (not illustrated) was seen irregularly dispersed in matrix and cells, throughout both the cartilage and "osteoid-like" tissues. A detailed description and analysis of the metachromatic behavior of the tissues will not be given at this time.

Chemistry

Results of analysis are summarized in Table I. A more detailed presentation will appear in a separate publication elsewhere. The yield of acid mucopolysaccharide, corrected for moisture, was 2.5% of the acetone-dried weight of the tissues. The glucosamine present was probably due to a trace contaminant. The uronic acid value was close to unity within the range of variability in colorimetric determinations. The excess of nitrogen over unity was accounted for by residual peptide bound to the polysaccharide, represented mainly by serine and glycine.. The sulfate content was unusually high. The preparation was hydrolyzed much more slowly by testicular hyaluronidase than was chondroitin sulfate of mammalian cartilage. This difference in rates of hydrolysis was due to the preparation's high sulfate content, since the product of chemical desulfation by the method of Kantor and Schubert (1957) was rapidly hydrolyzed by the enzyme.

The contribution of the sulfate to the polyelectrolyte properties of the polysaccharide is indicated by electrophoretic mobilities on cellulose acetate relative to chondroitin sulfate of 1.05 at pH 7.0 and 1.25 at pH 3.0.

The infrared spectrum closely resembled that of chondroitin sulfate C, rather than chondroitin sulfate A of vertebrate cartilage, indicating primarily equatorial sulfate conformation (Mathews, 1958). A minor difference was revealed by a peak at 700 $cm.^{-1}$, possibly due to sulfate not present in chondroitin sulfate C. The number average molecular weight was determined by osmometry as near 10,000; the intrinsic viscosity was 0.32.

A 40.0-mg. net weight sample of the basilar endoskeletal complex had an acetone-dried weight of 10.1 mg., and contained 0.320 mg. of hydroxyproline.

DISCUSSION

It is somewhat surprising to find in an annelid an endoskeleton of such complexity as is seen in *Eudistylia*. Nevertheless such endoskeletons are not uncommon, as we have verified by examination of related polychaetes, including *Myxicola, Spirographis* and *Sabella;* and of course, as is known from references given in the opening paragraph of this paper.

The histology of these tissues will be discussed briefly from several standpoints. To begin with, the cartilage is of the cellular variety, possessing relatively thin seams of matrix. Such cartilages are seen amongst invertebrates in tissues such as the odontophore cartilages of gastropod molluscs, and the gill cartilage of young specimens of *Limulus polyphemus* (Person and Philpott, 1963a). Amongst vertebrates such cellular cartilages are also widely encountered as in the vertebrae of *Mustelus* (dogfish), ear and xiphisternal cartilage of the young white rat, *etc.* (see also Schaffer, 1930). The cellularity or relative lack of matrix (as compared with cartilages of the hyaline variety) permits a remarkably plant-like organization of *Eudistylia* cartilage in certain regions of its endoskeletal complex (see especially Figs. 6, 7 and 8).

The marked resemblances between plant tissues and cartilage have been of interest to biologists since the time of Schwann and Schleiden (Schwann, 1839), and seems to be forgotten and rediscovered in each generation [see Godman and Porter (1960) and Person and Philpott (1963b)]. It is worth stressing that while the correspondence of plant and animal cells and subcellular organelles has been recognized as a keystone for the understanding of cell form and function, still, at the tissue level of biological organization and above, there are few instances in which plant and animal structures can be compared, with the noteworthy exception of cartilage and plant tissues! Such correspondences may be either fortuitous or indicative of the existence, in cartilage and certain plant tissues, of important similarities in fundamental biochemical and physiological processes occurring at *tissue* levels. However, because there is so little communication between botanists and zoologists concerning biological processes at and above the tissue level of organization, and because these correspondences may have a deeper biological meaning, a fuller and more detailed study of the correspondences between plant and cartilage tissues is underway in our laboratory.

Also, Johnson (personal communication) has said that the "osteoid-like" tissue which forms part of the endoskeletal complex of *Eudistylia* is very similar in appearance to forms of osteoid seen in a variety of human skeletal tissue tumors.

Finally, consideration of the morphology of the skeletal tissues of *Eudistylia* leads to an intriguing possibility which may be of especial interest to students of the evolution of animal skeletal systems: If the cartilage and "osteoid-like" components of the skeletal complex of *Eudistylia* were mineralized, then one would have a structure strongly resembling the outer armor of the vertebrate ancestors, the ostracoderms. For example, in Figure 3, the tissue components *ep* and *ma,* if mineralized, might strongly resemble enamel. Where the *ma* is penetrated by vascular channels to give the osteoid like-tissues, *os* (Fig. 5), and in regions where cartilage is found, the appearance of the hypothetically mineralized components could easily resemble many of the tissue components seen in dermal armor of the ostracoderms (see Gregory, 1951; Stensiö, 1927; Bystrow, 1959; Denison, 1963). It is to be definitely understood that we are *not* advocating that ostracoderms are derived from annelids. But the potential conversion of portions of *Eudistylia*'s skeletal system to an ostracoderm-like armor is most interesting, and merits further investigation.

The chemical, enzymatic and physico-chemical data indicate that the acid mucopolysaccharide of *Eudistylia* cartilage closely resembles chondroitin sulfate of mammalian cartilage in many respects, but differs in its higher sulfate content. Although most vertebrate cartilage chondroitin sulfates have sulfate: hexosamine ratios of 1:1, exceptionally high sulfate contents similar to those reported for *Eudistylia* are characteristic of preparations from cartilage of the coelacanth (*Latimeria*), members of the class of chondrichthyes, and also the hagfish (*Myxine*) (Mathews, in Press a). The presence of serine and glycine as the main residual amino acids suggests that the acid mucopolysaccharide of *Eudistylia* tissue may be covalently bound to peptide *via* serine hydroxyl (with an adjacent glycine residue) in a manner similar to that found for chondroitin sulfate of vertebrate cartilage (Mathews, in Press a). Also, the acid mucopolysaccharide level of *Eudistylia* cartilage is within the range of chondroitin sulfate levels of most vertebrate cartilages, which vary from 1.5% to 20%. Thus, *Eudistylia* endoskeletal tissue, while unusual with respect to the excess sulfate content of its acid mucopolysaccharide component, nevertheless falls within the range of biochemical criteria for vertebrate cartilage. In this connection, *Eudistylia* is also not unique among invertebrates, and resembles both *Loligo* and *Limulus,* whose cartilages also contain very similar chondroitin sulfates (Mathews, Duh and Person, 1962).

The high level of hydroxyproline, together with the histologic appearance and staining properties of the tissues, are strongly indicative of the presence of collagen in the endoskeletal tissues of *Eudistylia.* The value of 3.2% hydroxyproline is comparable to the value of 3.5% hydroxyproline in embryonic (18-day) chick articular cartilage (Mathews, in Press b).

The inability to obtain a gelatin which sets (when cooled) from invertebrate cartilages, was thought by Krukenberg (1882), Schaffer (1930) and others to constitute evidence that collagen was not present in the tissues. Invertebrate collagens are quite widespread in occurrence (Gross, 1963), but at the present time no worker has yet reported isolating a setting gelatin from any invertebrate connective tissue, so that the gelation phenomenon cannot be considered a necessary criterion for, or essential property of, invertebrate collagens. At present, the identification of collagen (invertebrate or vertebrate) also depends upon a characteristic wide-angle x-ray diffraction pattern (Gross, 1963). Such patterns are not yet available for *Eudistylia* tissues. Nevertheless, the presence of hydroxyproline in *Eudistylia* and other invertebrate cartilage tissues, as well as a characteristic light- and electron-microscope appearance (Person and Philpott, 1963a), makes it very likely that collagen is present. In the work reported by Person and Philpott (1963a) a collagen with 650 Å band space is shown in *Busycon* cartilage.

The inability of early workers to detect "mucins," *i.e.,* chondroitin sulfate-containing components, in polychaete cartilage (Krukenberg, 1882) and in other invertebrate cartilages (Schaffer, 1930) was also used as a major argument against the existence of cartilage tissues in invertebrates. With the advent of better methods for extraction and identification of these acidic polysaccharides, this argument is eliminated (present data; see also Mathews, Duh and Person, 1963, for data on chondroitin sulfates of *Limulus* and *Loligo* cartilages).

In view of the above, it is believed that the controversy over the existence or non-existence of "true" cartilage in invertebrates should be reopened, and should be answered in the affirmative because the invertebrate tissues in question: (1) are composed of cells suspended in a relatively rigid matrix of varying abundance, (2) are rich in acidic polysaccharides including chondroitin sulfates, and (3) have a high collagen content. The above criteria are also those by which vertebrate cartilages are designated.

Supported by grants from the U. S. Public Health Service, The National Foundation and The Chicago Heart Association.

LITERATURE CITED

Bystrow, A. P., 1959. The microstructure of skeleton elements in some vertebrates from the lower Devonian deposits of the U.S.S.R. *Acta Zool.,* **40**: 59–76.

Claparède, E., 1873. Recherches sur la structure des Annélides Sédentaires. H. Georg, Genève.

Crescitelli, F., 1945. A note on the absorption spectra of the blood of *Eudistylia gigantea* and of the blood pigment in the red corpuscles of *Cucumaria miniata* and *Molpadia intermedia. Biol. Bull.,* **88**: 30–36.

Denison, R. H., 1963. The early history of the vertebrate calcified skeleton. *Clin. Orthopaed.,* **31**: 141–151.

Evenkamp, H., 1931. Morphologie, Histologie und Biologie der Sabelliden-specified *Laonome kroyeri* Malmyn und *Euchone papillose* M. Sars. *Zool. Jahrb. Jena, Abt. Anat.,* **53**: 405–534.

Godman, G. C., and K. R. Porter, 1960. Chondrogenesis, studied with the electron microscope. *J. Biophys. Biochem. Cytol.*, **8**: 719–760.

Gregory, W. K., 1951. Evolution Emerging. Vol. II, Figs. 6.2; 6.3; 6.4; 6.8. Macmillan Co., New York.

Gross, J., 1963. Comparative biochemistry of collagen. *In*: Comparative Biochemistry, ed. by M. Florkin and H. S. Mason, Vol. 5 Part C, pp. 307–346. Academic Press, New York.

Hempelmann, F., 1928–34. *In*: Handbuch der Zoologie, ed. by W. Kükenthal and T. Krumbach, Zweite band, Zweite Hälft, pp. 1–212, DeGruyter and Co., Berlin.

Hyman, L., 1940. The Invertebrates. Vol. 1. McGraw Hill Book Co., New York.

Kantor, T. G., and M. Schubert, 1957. A method for the desulfation of chondroitin sulfate. *J. Amer. Chem. Soc.*, **79**: 152–153.

Krukenberg, C. F. W., 1882. Vergleichend-Physiologische Studien, pp. 49–53. C. Winter, Heidelberg.

Lash, J. W., 1959. The presence of myoglobin in the cartilage of a marine snail, *Busycon*. *Science*, **130**: 334.

Mathews, M. B., 1958. Isomeric chondroitin sulfates. *Nature*, **181**: 421–422.

Mathews, M. B., In Press a. The molecular evolution of cartilage. *Clin. Orthopaed.*

Mathews, M. B., In Press b. Chondroitin sulfate and collagen in inherited skeletal defects of chickens. *Nature.*

Mathews, M. B., J. Duh and P. Person, 1962. Acid mucopolysaccharides of invertebrate cartilage. *Nature*, **193**: 378–379.

Mathews, M. B., and S. Glagov, 1966. Mucopolysaccharide patterns in ageing human cartilage. *J. Clin. Invest.*, **45**: 1103–1111.

Nichol, E. A. T., 1930. The feeding mechanism, formation of the tube and physiology of digestion in *Sabella pavonina*. *Proc. Roy. Soc. Edinburgh*, **56**: 537–598.

Nowikoff, W., 1912. Studien über das Knorpelgewebe von Wirbellosen. *Zeitschr. f. wiss. Zool.*, **103**: 661–717.

Person, P., and A. Fine, 1957. Observations on the histology and oxidative metabolism of gill cartilage from *Limulus polyphemus*. *Biol. Bull.*, **113**: 331.

Person, P., and D. E. Philpott, 1963a. Invertebrate cartilage. *Ann. N. Y. Acad. Sci.*, **109**: 113–126.

Person, P., and D. E. Philpott, 1963b. Limulus gill cartilage: a "plant-like" tissue. *Biol. Bull.*, **125**: 355–356.

Pritchard, J. J., 1956. General anatomy and histology of bone. *In*: The Biochemistry and Physiology of Bone, ed. by G. H. Bourne, pp. 1–23, Academic Press Inc., New York.

Raven, Chr. P., 1958. Morphogenesis in Molluscs. Pergamon Press, New York.

Romer, A. S., 1942. Cartilage, an embryonic adaptation. *Amer. Naturalist*, p. 394.

Romer, A. S., 1964. Bone in early vertebrates. *In*: Bone Biodynamics, ed. by H. M. Frost, pp. 13–37. Little, Brown & Co., Boston, Mass.

Schaffer, J., 1913. Über den feinen Bau und die Entwicklung des Knorpelgewebes und über verwandte Formen der Stutzsubstanz. IV. *Zeitschr. f. wiss. Zool.*, **105**: 280–347.

Schaffer, J., 1930. Die Stutzgewebe. *In*: Handbuch der Mikroskopischen Anatomie des Menschen, ed. by W. von Möllendorf, Zweiter Band, Zweiter Teil, pp. 1–390. Julius Springer, Berlin.

Schwann, T., 1839. Mikroskopische Untersuchungen ueber die Ubereinstimmung in der Struktur und dem Wachstum der Tiere und Pflanzen. Berlin.

Stegemann, H., 1958. Mikrobestimmung von Hydroxyprolin mit Chloramin T und p-Dimethylaminobenzaldehyd. *Zeitschr. Physiol. Chem.*, **311**: 41–45.

Stensio, E., 1927. The Downtownian and Devonian vertebrates of Spitzbergen. Part I. Family Cephalaspidae. *Skrift Svalbard Nordishavet, no. 12*: 1–391.

Thomas, J. G., 1940. *Pomatoceros, Sabella* and *Amphitrite* (Liverpool Marine Biol. Comm. Memoir No. 33). *Proc. Trans. Liverpool Biol. Soc.*, **43**: 1–88.

Viallanes, M. H., 1885. Sur la structure de la squellette branchiale de la Sabelle. *Ann. Sci. Nat. Paris, Ser.* 6, **20**(2): 1–20.

THE RELATIONSHIP OF TEMPERATURE TO THE LARVAL DEVELOPMENT OF NASSARIUS OBSOLETUS (GASTROPODA)[1, 2]

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Temperature has long been suggested as an important factor regulating the developmental rate, length of pelagic life, and mortality of larvae from benthic marine invertebrate organisms. It is known, for example, that the rate of early cleavage, within certain limits, is related directly to water temperature (*vide* Costello *et al.,* 1957). There have been a number of attempts by marine biologists, especially with species of economic value, to relate the success of settlement during any specific year to the sea water temperature at the time of larval development. Among bivalve mollusks, the oysters *Crassostrea virginica* Gmelin and *Ostrea edulis* L. and the clam *Venus mercenaria* L. have particularly been studied (*e.g.,* Needler, 1940; Medcof, 1939; Korringa, 1952; Carriker, 1961, pp. 212–213).

Seno, Hori and Kusakabe (1926) determined the effect of temperature on the early development of *Ostrea gigas* from the time of fertilization to the early shelled larva. Clark (1935) examined the effect of reduced temperature on the early development of *Crassostrea virginica.* Not until the development of adequate techniques for growing larvae in mass culture from fertilization to settlement (Allen and Nelson, 1911; Bruce *et al.,* 1940) has it been possible to examine experimentally the relationship of temperature to the development of molluscan larvae. Loosanoff *et al.* (1951) and Loosanoff (1959) were the first to demonstrate successfully in the laboratory the role of temperature throughout the entire period of pelagic larval development of the bivalve mollusk, *Venus mercenaria.* Subsequently studies of comparable detail have been made by Walne (1958) with *Ostrea edulis;* by Davis and Calabrese (1964) with *Crassostrea virginica;* by Stickney (1964) with *Mya arenaria* L.; and by Bayne (1965) with *Mytilus edulis* L. The very interesting research on gastropod larvae by Lebour (1937) did not include experimental work using mass culture techniques, as her primary concern was the identification and description of veligers from the plankton. Similarly, Thorson (1946, 1950) has followed the development of gastropod larvae by examining plankton tows periodically taken from the Øresund, but he has not attempted to undertake laboratory culture work as a means of understanding the relative importance of environmental factors on pelagic larval development. It is the purpose of this paper to describe such a laboratory study using the common marine intertidal prosobranch gastropod *Nassarius obsoletus* Say.

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[1] Contribution No. 1815 from the Woods Hole Oceanographic Institution, Woods Hole, Massachusetts.

[2] This research was supported in part by Grants 17883 and GB-2207 from the National Science Foundation. I wish to thank my research assistant, Mr. Gordon Enk, for his help during the conduct of some of the experiments here described.

Nassarius obsoletus inhabits marine and estuarine intertidal flats from Chaleur Bay in the Gulf of St. Lawrence to Cape Kennedy (Canaveral) in northern Florida. Although the early cleavage stages of development are well known to embryologists (*vide*: Clement, 1962; Thompson, 1955), the later planktotrophic veliger larvae were not described until recently (Scheltema, 1962a). Experimental studies have determined the role of salinity in larval survival and development (Scheltema, 1962b, 1965). Some mechanisms which control the length of pelagic life and the delay of metamorphosis are also known from previous experiments (Scheltema, 1961). Aspects of the ecology of the adults have been discussed by Dimon (1905), Jenner (1956a, 1957), and Scheltema (1964).

This study is divided into two parts: (1) the relationship of temperature to the rate of early embryological development within the egg capsule, as indicated by the time required for emergence of veliger larvae into the sea; (2) the relationship of temperature to growth and length of planktonic larval life. Before giving an account of the experimental work, however, I shall describe briefly the reproductive habits of *N. obsoletus,* as these have not previously been recorded in any detail.

Reproduction and Spawning

The onset of spawning in *Nassarius obsoletus* differs with latitude and is directly related to sea water temperature. As the species inhabits an environment where temperature can be highly variable over short periods, the exact timing of reproduction is never very precise. It can be shown, for example, that at Beaufort, North Carolina, the water temperature on the intertidal flats in early February may differ as much at 5° C. between high and low tide; a change from 13° to 17° C. has been recorded in the area on the flats where females of *N. obsoletus* occur. At Barnstable Harbor, Cape Cod, Massachusetts, the low-water temperature on the flats inhabited by *N. obsoletus* increases abruptly in a period of about two to three weeks from 13° C. in mid-May to 23° C. in early June. That the females respond to elevation of water temperature by spawning can easily be shown by bringing snails into the laboratory during mid-winter months. Under such conditions, when the animals are fed, spawning commences within a week. Copulation occurs during the same period as spawning.

Under natural conditions the process of gametogenesis is completed long before the normal time for spawning. This is known from frequent anatomical and histological examination of snail gonads throughout the period extending between the cessation of spawning and the completion of gametogenesis. Three geographically separated populations were followed: (1) Barnstable Harbor, Cape Cod, Massachusetts; (2) Beaufort, North Carolina; and (3) Charleston, South Carolina, the latter two in somewhat less detail than the former. In the northern end of the range, gametogenesis usually proceeds within six weeks after the cessation of spawning, that is, sometime during late September. However, in the southern end of the range, spawning is completed by mid-June (Jenner 1956b), but the onset of gametogenesis is apparently delayed for several months. This delay needs confirmation by more frequent observations. There is no question, however, that both in New England and at Beaufort, North Carolina, gametogenesis has been completed by late fall, *i.e.,* mid- to late November. The attainment of sexual competence can readily be determined externally in the living intact organisms by the structure of the penis in the male and by the pigmentation at the end of the oviduct in the female.

Natural spawning normally begins in February at the southern end of the species range, about mid- to end-April in Delaware Bay and the south shore of Cape Cod, and early June in Cape Cod Bay and Maine. Consequently, gametogenesis is completed almost six months before the natural spawning of populations found north of Cape Cod and at least two months before spawning in populations south of Cape Hatteras, North Carolina. Ecologically it is doubtlessly advantageous for the species to spawn as soon as the sea water becomes warm enough for larval development and the early gametogenesis allows great flexibility in the time of spawning.

The egg capsules of *N. obsoletus* are deposited on any solid object on the intertidal flats, *e.g.*, shells, *Diopatra* tubes, thallus algae, *etc.* Ankel (1929) has described in detail the deposition of egg capsules by the European species, *Nassarius reticulatus* L., and this account agrees in every essential detail with the process as it occurs in *N. obsoletus*. A description of the egg capsules of *N. obsoletus,* along with the characteristics distinguishing them from other members of the genus found along the east coast of the United States, has been given by Scheltema (1962a) and by Scheltema and Scheltema (1965).

Relationship of Temperature to Embryonic Development and the Escape of Larvae from the Egg Capsule into the Sea

The larvae of *Nassarius obsoletus* after the completion of their embryonic development emerge through an opening at the free end of the egg capsule. The precise method by which the opening is made by the larvae at the time of their escape is not understood, but its position at the distal end is structurally pre-determined at the time of capsule formation.

The relationship of temperature to the time required between spawning and emergence of veligers from their egg capsules can be demonstrated by a simple experiment. Adults of *N. obsoletus* readily lay egg capsules upon the sides of aquaria. If, shortly after their deposition, several hundred egg capsules are collected and placed at regular temperature intervals, falling within the extreme range at which they are normally found in nature, the effect of temperature on development can be determined. A number of such experiments were performed at temperature intervals of 28°, 19.5°, 16.5° and 11.5° C. Egg capsules laid within a 48-hour period were collected from snails that had been actively laying for several weeks. For the purpose of the experiment, the median age of the egg capsules was considered to be 24 hours. The exact time of deposition of each capsule is not particularly meaningful as the degree of development of the eggs within each capsule is known to vary at the time of attachment. Early in the spawning period, capsules are occasionally retained within the oviduct of the female until development of the embryos is almost completed to the veliger stage, but as the season of spawning proceeds there is normally little delay between the initiation of embryological development and egg capsule attachment. The rates of development at different temperatures were observed simultaneously from random aliquots taken from the same "harvest" of egg capsules. At the beginning of the experiment each capsule was examined to make certain that it was intact and had not been damaged during its removal from the walls of the aquarium. Between 250 and 300 egg capsules were used at each temperature. Starting with the time at which larval emergence first began, the number of empty egg capsules in each container was determined at frequent intervals.

The results obtained in one such experiment are shown by the series of curves in Figure 1, in which the ordinate gives the cumulative percentage of capsules from which larvae had emerged and the abscissa the number of days since the deposition of the capsules. The curves represent development at each of the different temper-

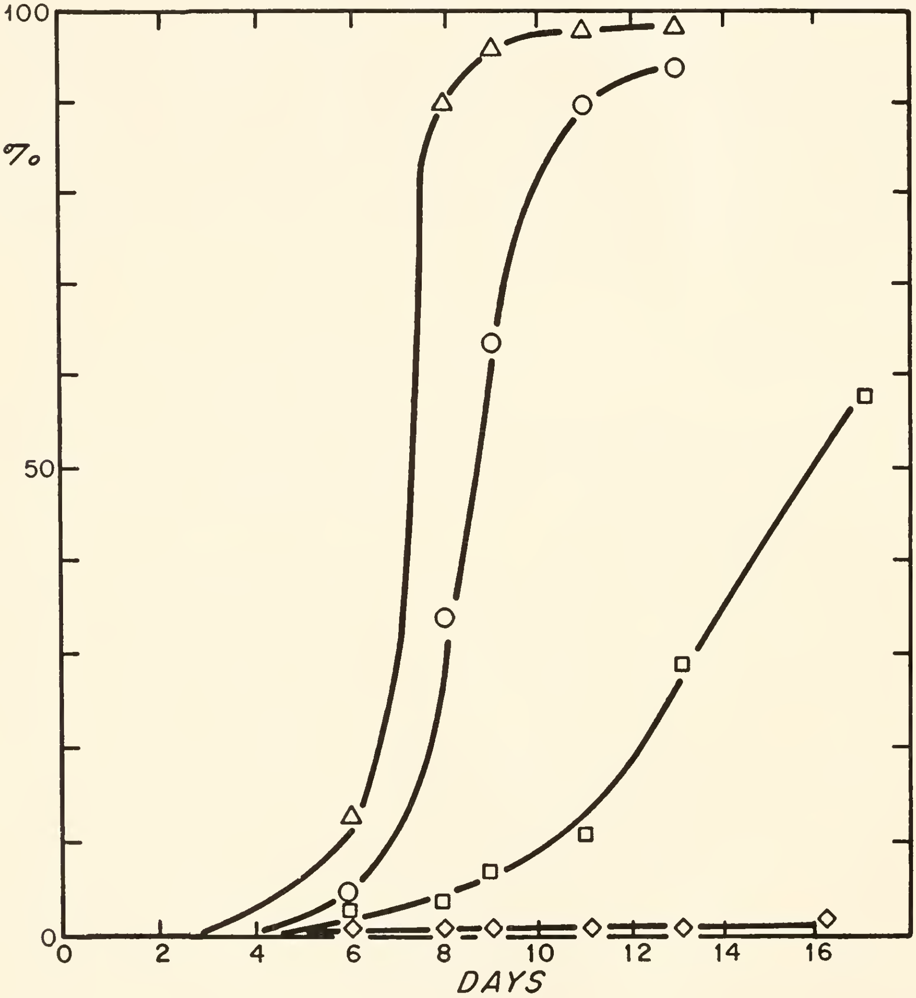

FIGURE 1. Percentage egg cases of *Nassarius obsoletus* from which larvae have emerged relative to the time since spawning occurred. The curves represent development at four different temperatures: 28.0° C. (△); 19.5° C. (○); 16.5° C. (□); and 11.5° C. (◇). The abscissa gives the time in days since the deposition of egg capsules; the ordinate is the cumulative percentage of egg capsules from which larvae have emerged. The values along the abscissa are approximate (± one day) as the egg capsules were laid over a 48-hour period.

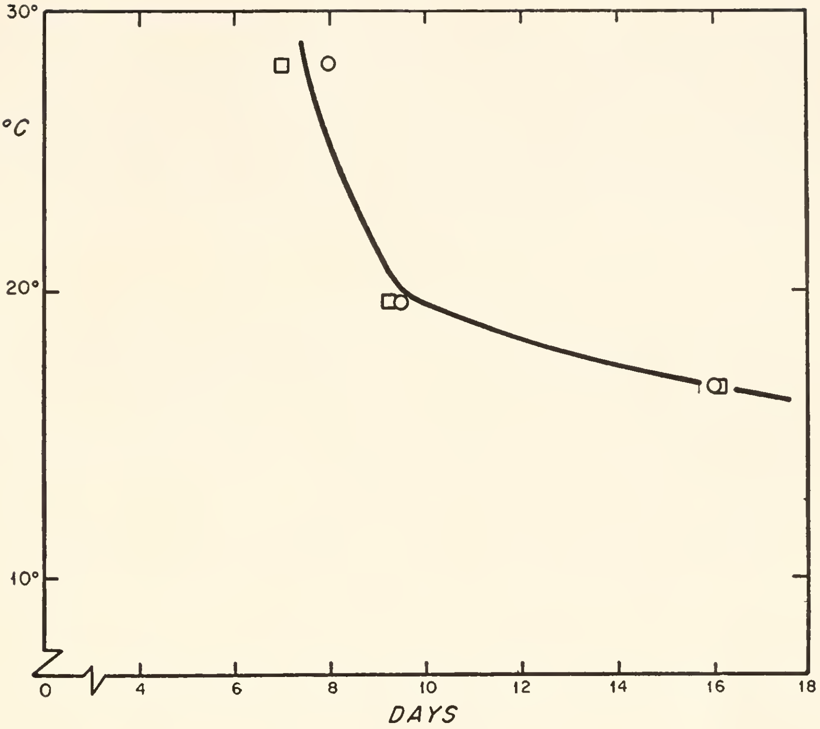

Figure 2. Time in days required between spawning and emergence of *Nassarius obsoletus* larvae from egg capsules as a function of temperature (° C.). The points indicate the number of days necessary for emergence of 50% of the larvae. Results are from two geographically isolated regions, Beaufort, North Carolina (○) and Cape Cod, Massachusetts (□). No significant difference is discernible in the results between egg capsules obtained from the two populations. The data are derived from experiments shown in Figure 1, and from similar experiments using egg capsules from snails obtained from Beaufort, North Carolina. From 250 to 300 egg capsules were used at each temperature in experiments with the Cape Cod populations. Approximately 100 egg capsules were used at each temperature in the Beaufort experiments.

atures. No larvae emerged from the capsules held at 11.5° C. during the course of the experiment. It has previously been shown that at a temperature between 11° and 13° C., embryos do not complete their development but that a large proportion remain viable for a period of up to at least nine weeks (Scheltema, 1962a). When returned to warmer water such embryos developed normally.

The relationship between temperature and the time required for the emergence of veliger larvae from the egg capsules is best understood by reference to Figure 2, where the number of days required for the liberation of veliger larvae from the first 50% of the capsules is indicated along the abscissa, and the temperature (° C.) at which the development took place is shown along the ordinate. Whereas the time required for emergence increases slightly between 28° and 20° C., about 0.25 day per degree centigrade, it increases more rapidly at temperatures below 20° C.; between 20° and 16.5° C., there was an increase of two days for each degree of lowering of the temperature. The figure also shows that there is no significant difference in the effect of temperature on egg development in populations of snails from Beaufort, North Carolina, and from Cape Cod, Massachusetts. These results differ from those of Dehnel (1955) obtained from several intertidal species of gastropods along the west coast of North America. He found that when embryos collected from different geographical regions were allowed to cleave at an identical temperature, there was a clear difference in the developmental rate; the relationship appeared to be clinal.

The Relationship of Temperature to Growth Rate

A method for obtaining large numbers of *Nassarius obsoletus* veliger larvae and for growing mass cultures to be used in experimental work has already been described (Scheltema, 1962a). The cultures used in the present experiments were 10 liters in volume, each containing from 5000 to 10,000 larvae. Food used throughout the duration of these experiments was *Phaeodactylum tricornutum* Bohlin which was obtained from unialgal cultures.

Larvae which had emerged from a large number of egg capsules over a 24-hour period were divided equally among 10-liter containers. The number of larval cultures started was determined by the number of temperatures at which growth was to be measured. A sample of the larvae was also taken at the beginning of each experiment so that the initial size after emergence from the egg capsule could be determined. Each larval culture was fed an identical quantity of food (*ca.* 200,000 cells/ml.) from the same unialgal culture of *P. tricornutum.* This amount of food was great enough so that a slight excess remained after three days.

A sample of from 50 to 100 larvae was removed from each culture every third day. At this time the water was also changed and new food cells were added. The aliquot of larvae was preserved in 70% alcohol for later measurement.

The growth of larvae was estimated by measuring the shell length of 35 specimens picked randomly from the larger aliquot described above. An ocular micrometer at a magnification of 100 × was used. The longest dimension of the shell of a larva was considered to be the length.

The temperature of the cultures was maintained by means of water baths improvised from commercial soft drink coolers. The maximum deviation from the stated mean was 1.5° C., but the mean deviation was only ±0.5° C. Because all the experiments extended over more than two weeks, these variations were not considered serious.

In the first series of experiments, larvae were grown simultaneously at either three or four different temperatures. The results from one representative experiment are shown in Figure 3, where the mean temperatures were 16.5°, 21.0°, 24.8° and 29.5° C. From this experiment it was concluded that the optimum temperature for growth under laboratory conditions was approximately 25° C. This was further verified in three other experiments. At either higher or lower temperatures the growth was significantly less. That the rate of growth is not uniform throughout larval development, particularly at optimum temperatures, can also be seen in Figure 3. The lowest temperature at which larvae successfully grew to metamorphosis was between 16° and 17° C.

To determine the maximum effect of temperature upon growth I made a second series of experiments. The growth rate at approximately 25° C., an optimum temperature, was compared with that at 17.5° C., a value near the lowest temperature at which larval development is completed to settlement. The results of one such series of experiments are shown in the growth curves in Figure 4. Here the upper cumulative growth curve is from larvae grown at 25.2° C.; the lower curve represents growth under similar conditions except that the temperature was 17.5° C. The minimum length at which the veliger larvae have been shown to metamorphose lies between 550 and 600 μ, but the median size is near 700 μ. On the graph in Figure 4 the inflection points on both curves are at approximately 600 μ. In order to compare growth rates between two temperatures it is clearly necessary to consider only those portions of the curves which precede the points of inflection. After the median size for metamorphosis is reached (*i.e.*, 700 μ) relatively little further growth occurs. The maximum recorded size at metamorphosis is 950 μ, but this size is rarely attained by larvae. The length of the period following the completion

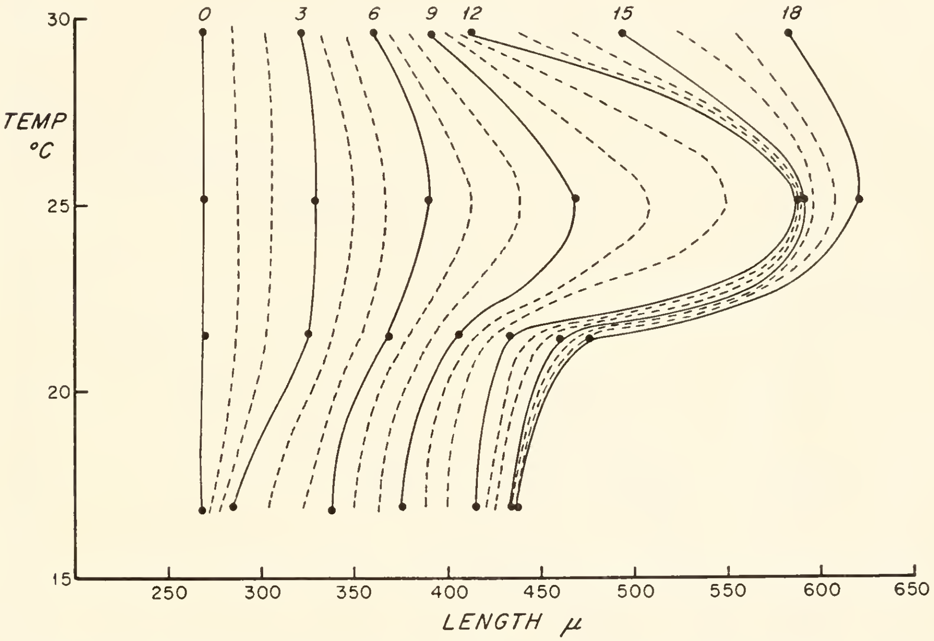

FIGURE 3. The effect of temperature upon the growth of the planktonic veliger larvae of *Nassarius obsoletus*. The ordinate gives the temperature range over which the larvae were grown. The abscissa gives the length attained by the larvae. Each curve shown by a solid line represents the total cumulative growth completed by the larvae within the number of days indicated by the numeral over the curve. The amount of growth for any length of time and for any temperature which was tested can be easily derived from the figure. The points on the solid lines represent the actual experimental values obtained. Curves given with dashed lines represent the average growth at intervening days and were derived by linear interpolation.

of development (*i.e.*, the attainment of 700 μ) is primarily dependent on a settlement response of the larvae. This is further discussed below.

All the experiments, including those of the first series above for which no data have thus far been given, are summarized in Table I. The data from all these

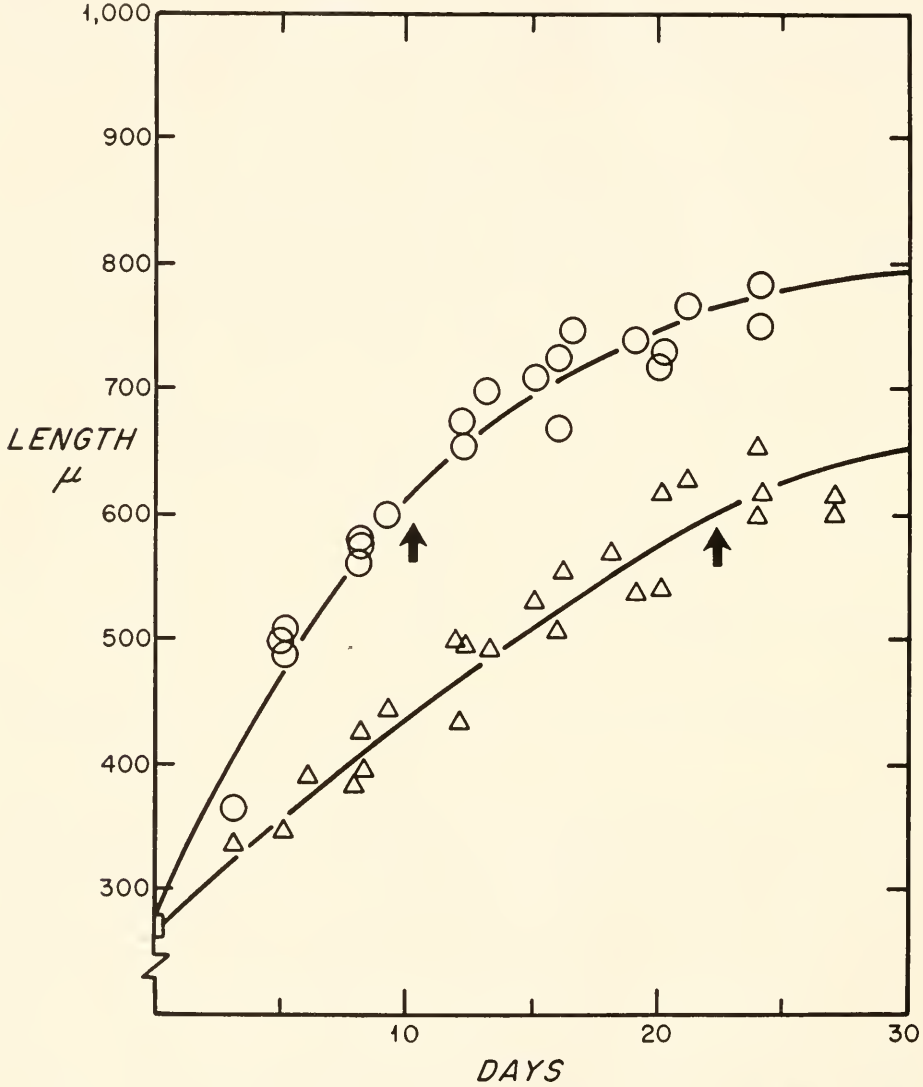

FIGURE 4. Cumulative growth curves of the planktonic veliger larvae of *Nassarius obsoletus*. The upper curve (○) represents cumulative growth at an average temperature of 25.2° C.; the lower curve (△) represents growth of larvae at 17.5° C. The attainment of the "creeping-swimming" stage is indicated on each curve by an arrow. This shows the end of the "developmental period" to the left of arrow and the beginning of the "delay period" to the right of arrow. Note that the "developmental period" is approximately twice as long at 17.5° C. (21 days) as it is at 25.2° C. (10 days).

experiments cannot be directly compared because (1) the larvae were not randomly obtained from the same parents and therefore are not known to be genetically similar, and (2) the experiments were not conducted simultaneously, using the same algal culture, so that the quality of the food was not necessarily the same. However, the data can be pooled and the results compared by using the value I which is the *per cent inhibition of growth* (Scheltema, 1965). This may be computed from the equation

$$I = \frac{\triangle A - \triangle B}{\triangle A} \times 100$$

where $\triangle A$ is the change in length of the shell between the beginning and end of an experiment at a temperature optimum for growth (*ca.* 25° C.) and $\triangle B$ is the change in shell length at a minimum temperature required for the completion of development.

TABLE I

The growth of Nassarius obsoletus larvae at a near optimal temperature and at a minimum temperature required for completion of development, showing the maximum inhibition attributable to temperature

Expt. no.	Age in days at end of expt.	Length μ at begin. expt. (a)	Mean temp. °C.	Length** μ at end expt. (A)	Growth μ (ΔA)	Mean temp. °C.	Length** μ at end expt. (B)	Growth μ (ΔB)	Per cent inhibition of growth $\frac{\Delta A - \Delta B}{A} \times 100$
I	12	280*	25.1	657± 6	377	17.3	436±5	156	59
II	12	280*	25.0	655± 8	375	17.2	498±8	218	42
III	13	280*	24.5	698±10	418	17.7	488±7	209	50
IV	12	268	26.4	569± 5	301	17.5	496±5	228	24
V	12	268	26.4	672± 7	404	17.3	492±5	234	42
VI	17	271	23.9	615±11	344	15.8	447±7	176	49
VII	12	262	23.8	529± 7	267	15.9	422±8	160	40
VIII	9	268	24.6	463± 5	195	16.5	361±5	93	52
IX	12	268	25.3	589± 7	321	16.6	413±6	145	55
									mean = 46

* Estimated values.
** One standard error of the mean is indicated.

The value of $\triangle A$ is determined by subtracting the initial length of the veliger larvae at the beginning of the experiment from the length attained when the experiment was terminated. Hence

$$\triangle A = (A - a)$$

where a is the initial length at the time the larvae emerged from the egg capsule and A is the final length of the larvae when grown at 25° C. Similarly $\triangle B$ is obtained by subtracting the initial length a, from B where B is the final length of the larvae grown at around 16° or 17° C. The time at which each experiment was terminated was determined by the inflection point on that curve which represented the culture having optimum growth (*i.e.,* 25° C.).

With a single exception the values of I fall between 40% and 60% and the mean *per cent inhibition of growth* attributable to temperature was approximately 46%. This represents the average maximum-difference which can be accounted for by temperature alone.

Discussion

As the onset of spawning by *Nassarius obsoletus* is dependent upon temperature, its timing is never precise. Gametogenesis is always completed several months before spawning occurs, and consequently a short period of warming can very easily initiate spawning. Such conditions occur when the low tide falls near noon on clear sunny days during early spring. Experimental evidence now shows, however, that embryos can survive over long periods in cold water, but at a sharply reduced developmental rate. Somewhat similar results have been obtained with *Nassarius reticulatus* from the Black Sea (Bekman, 1941). It is very unlikely, from experimental evidence, that embryonic development of *N. obsoletus* into free-swimming veliger larvae is ever completed, under conditions in nature, before the water temperature rises high enough to insure the completion of pelagic development.

Although an optimum growth of planktonic larvae in the experiments occurred at 25° C., it is not clear whether this was an intrinsic characteristic of the veligers themselves or whether growth was indirectly influenced by the effect of temperature on the algal food. *Phaeodactylum tricornutum* does not long survive at temperatures above 25° C. However, as the larvae were fed fresh algal cells every third day and since an excess always remained in suspension at the end of this period, it was believed that this effect must have been minimal. It is not possible, however, to rule out such an indirect factor in the experimental results. Davis and Calabrese (1964) have suggested that enzymes required to digest naked flagellates are active at much lower temperatures than those involved in the digestion of certain other food forms having thick cell walls. Very few dinoflagellates or diatoms can grow and survive equally well at temperatures of 15° and 30° C.; both their numbers and food value to larvae may differ markedly at either of these extremes. It is necessary, when relating experiments from the laboratory to natural conditions, to take into account the effect of temperature on the principal phytoplankton organisms upon which the larvae are likely to be feeding.

The length of pelagic larval life in some bivalves seems to be directly related to the temperature and growth rate. Thus Loosanoff (1959) has shown that the increase in pelagic larval life of *Venus mercenaria* is directly related to the decrease in temperature. However, the results are somewhat obscured because the criterion used to determine the length of larval life was the number of days required for settlement to first begin. Such a criterion largely neglects the effect of a delay in settling due to the lack of a desirable substratum, if indeed *Venus mercenaria* has such a delay. Davis and Calabrese (1964, p. 648) have shown that in *Crassostrea virginica* the last larvae to settle in their cultures usually have a planktonic life almost two times as long as the earliest veligers to metamorphose. Bayne (1965) has demonstrated that *Mytilus edulis* in the absence of an adequate substratum for settlement delays metamorphosis, and that this delay is accompanied by a gradual decrease in growth rate to zero.

The larval life of *Nassarius obsoletus* can be divided into two periods. The first of these is one of rapid growth and morphological development and will be termed the "developmental period." This is followed by a second period, the "delay period," during which there is a gradual decrease in growth. The "developmental period" ends at the inflection point on the cumulative growth curve (Fig. 4). External morphological development has been completed to the creeping-swimming stage (Scheltema, 1962a). The growth rate during the "developmental period" is essentially constant if the environment remains reasonably so. Two physical factors that are important in determining the slope of the growth curve, and consequently the length of the "developmental period." are temperature and, under certain circumstances, the salinity of sea water (Scheltema, 1965). At the end of the "developmental period," metamorphosis first becomes possible. The "delay period" which follows may vary greatly in its length. Its duration is largely determined by the availability of the bottom sediment favorable for further post-larval life. The evidence for delay in settlement and a response to bottom sediment in *N. obsoletus* has already been given in previous papers (Scheltema, 1956, 1961).

SUMMARY

1. Development of the embryos of *Nassarius obsoletus* within egg capsules is regulated by sea-water temperature. An increase in the time required between spawning and the emergence of veliger larvae is slight between 28° and 20° C., about 0.25 day for each degree decrease in temperature. Between 20° and 16.5° C., the corresponding increase was 2 days per degree decrease in temperature. At 11.5° C., development was not completed and larvae did not emerge from their egg capsules after nine weeks. However, a large proportion of these embryos survived and developed normally through metamorphosis when placed at room temperature.

2. The growth rate of planktonic veliger larvae of *N. obsoletus* was greatest at approximately 25° C. The lowest temperature at which the development to metamorphosis was completed was at 16° to 17° C. There was a 46% inhibition in the growth rate of larvae between the optimum temperature and the minimum temperature at which development is completed.

3. The larval life of *N. obsoletus* veligers may be divided into two stages. The first of these, the "developmental period," is one during which rapid growth and morphological development occur. This is followed by the "delay period" characterized by a gradual decrease in growth rate. Reduced temperature may influence the rate of growth and consequently the length of the "developmental period." The termination of the "developmental period" comes with the "creeping-swimming stage." The duration of the "delay period" may be quite variable and is determined by the availability of a favorable sediment for settlement.

LITERATURE CITED

ALLEN, E. J., AND E. W. NELSON, 1911. On the artificial culture of marine organisms. *J. Mar. Biol. Assoc.*, **8**: 421–474.

ANKEL, W. E., 1929. Über der Bildung der Eikapsel bei *Nassa*-arten. *Verhandl. Deutsch. Zool. Gesellschaft, Zool. Anz. (Suppl. Band)*, **4**: 219–230.

BAYNE, B. L., 1965. Growth and the delay of metamorphosis of larvae of *Mytilus edulis* L. *Ophelia* (Copenhagen), **2**: 1–47.

BEKMAN, M. J., 1941. On the biology of the marine gastropod, *Nassa reticulata* (Monter.) and *Nassa (Cydonassa) neritea*. *Bull. Acad. Sci. U.R.S.S. (Ser. Biol.)*, **3**: 347–352 (in Russian).

BRUCE, J. R., M. KNIGHT AND M. W. PARKER, 1940. The rearing of oyster larvae on an algal diet. *J. Mar. Biol. Assoc.*, **24**: 337–374.

CARRIKER, M. R., 1961. Interrelation of functional morphology, behavior, and autecology in early stages of the bivalve, *Mercenaria mercenaria*. *J. Elisha Mitchell Sci. Soc.*, **77**: 168–241.

CLARK, A. E., 1935. Effects of temperature and salinity on early development of the oyster. *Prog. Rept., Atl. Biol. St., St. Andrews, N. B.*, **16**: 10.

CLEMENT, A. C., 1962. Development of *Ilyanassa* following the removal of the D macromere at successive cleavage stages. *J. Exp. Zool.*, **149**: 163–216.

COSTELLO, D. P., M. E. DAVIDSON, A. EGGERS, M. H. FOX AND C. HENLEY, 1957. Methods for Obtaining and Handling Marine Eggs and Embryos. Marine Biological Laboratory, Woods Hole, 247 pp.

DAVIS, H. C., AND A. CALABRESE, 1964. Combined effects of temperature and salinity on development of eggs and growth of larvae of *M. mercenaria* and *C. virginica*. *Fishery Bull. (U. S. Fish and Wildl. Ser.)*, **63**: 643–655.

DEHNEL, P. A., 1955. Rates of growth of gastropods as a function of latitude. *Physiol. Zoöl.*, **28**: 115–144.

DIMON, A. C., 1905. The mud snail: *Nassa obsoleta*. *Cold Spring Harbor Monogr.*, **5**: 1–48.

JENNER, C. E., 1956a. A striking behavioral change leading to the formation of extensive aggregations in a population of *Nassarius obsoletus*. *Biol. Bull.*, **111**: 291.

JENNER, C. E., 1956b. The timing of reproductive cessation in geographically separated populations of *Nassarius obsoletus*. *Biol. Bull.*, **111**: 292.

JENNER, C. E., 1957. Schooling behavior in mud snails in Barnstable Harbor leading to the formation of massive aggregations at the completion of sexual reproduction. *Biol. Bull.*, **113**: 328–329.

KORRINGA, P., 1952. Recent advances in oyster biology. *Quart. Rev. Biol.*, **27**: 266–308; 339–365.

LEBOUR, M. V., 1937. The eggs and larvae of the British Prosobranchs with special reference to those living in the plankton. *J. Mar. Biol. Assoc.*, **22**: 105–166.

LOOSANOFF, V. L., 1959. The size and shape of metamorphosing larvae of *Venus (Mercenaria) mercenaria* grown at different temperatures. *Biol. Bull.*, **117**: 308–318.

LOOSANOFF, V. L., W. S. MILLER AND P. B. SMITH, 1951. Growth and setting of larvae of *Venus mercenaria* in relation to temperature. *J. Mar. Res.* (New Haven), **10**: 59–81.

MEDCOF, J. C., 1939. Larval life of the oyster (*Ostrea virginica*) in Biddeford River. *J. Fish. Res. Bd. Canada.*, **4**: 287–301.

NEEDLER, A. W. H., 1940. Helping oyster growers to collect spat by predicting sets. *Progr. Rept. Atl. Biol. Sta.*, **27**: 8–10.

SCHELTEMA, R. S., 1956. The effect of substrate on the length of planktonic existence in *Nassarius obsoletus*. *Biol. Bull.*, **111**: 312.

SCHELTEMA, R. S., 1961. Metamorphosis of the veliger larvae of *Nassarius obsoletus* (Gastropoda) in response to bottom sediment. *Biol. Bull.*, **120**: 92–109.

SCHELTEMA, R. S., 1962a. Pelagic larvae of New England intertidal gastropods. I. *Nassarius obsoletus* Say and *Nassarius vibex* Say. *Trans. Amer. Microsc. Soc.*, **81**: 1–11.

SCHELTEMA, R. S., 1962b. Environmental factors affecting length of pelagic development in the gastropod, *Nassarius obsoletus*. *Amer. Zool.*, **2**: 445.

SCHELTEMA, R. S., 1964. Feeding and growth in the mud-snail *Nassarius obsoletus*. *Chesapeake Sci.*, **5**: 161–166.

SCHELTEMA, R. S., 1965. The relationship of salinity to larval survival and development in *Nassarius obsoletus* (Gastropoda). *Biol. Bull.*, **129**: 340–354.

SCHELTEMA, R. S., AND A. H. SCHELTEMA, 1965. Pelagic larvae of New England intertidal gastropods. III. *Nassarius trivittatus*. *Hydrobiologia*, **25**: 321–329.

SENO, H., J. HORI AND D. KUSAKABE, 1926. Effects of temperature and salinity on the development of the eggs of the common Japanese oyster, *Ostrea gigas* Thunberg. *J. Fish. Inst. Tokyo*, **22**: 41–47.

STICKNEY, A. P., 1964. Salinity, temperature and food requirements of soft-shell clam larvae in laboratory culture. *Ecology*, **45**: 283–291.

THOMPSON, J. R., 1955. The embryology and organogeny of the common eastern mud snail, *Nassarius obsoletus* Say. M. S. thesis, Duke Univ., Durham, North Carolina.

THORSON, G., 1946. Reproduction and larval development of Danish marine bottom invertebrates with special reference to the planktonic larvae in the sound (Øresund). *Medd. Komm. Danm. Fiskeriog Havunders. Ser. Plankton,* **4**: 1–523.

THORSON, G., 1950. Reproductive and larval ecology of marine bottom invertebrates. *Biol. Rev.*, **25**: 1–45.

WALNE, P. R., 1958. Growth of oysters (*Ostrea edulis* L.). *J. Mar. Biol. Assoc.*, **37**: 415–425.

THE MORPHOLOGY, LIFE-HISTORY, AND SYSTEMATIC RELATIONS OF THE DIGENETIC TREMATODE, UNISERIALIS BREVISERIALIS SP. NOV., (NOTOCOTYLIDAE), A PARASITE OF THE BURSA FABRICIUS OF BIRDS[1]

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The genus *Uniserialis* was erected by Mary Beverley-Burton (1958) to contain *Uniserialis gippyensis*, a new species from the intestinal caeca and bursae Fabricii of mallard ducks, *Anas platyrhyncha platyrhyncha* Linn., taken near Ipswich, Suffolk, England. The generic diagnosis stated, "Notocotylidae Lühe, 1909; body flattened, small, rather pointed at the anterior end but rounded posteriorly; cuticula aspinose. Ventral surface with a single median longitudinal row of sessile glands. Oral sucker terminal, pharynx absent; esophagus short leading to intestinal bifurcation, ceca simple ending blindly near posterior extremity. Ventral sucker absent. Common genital pore ventral, median, anterior to intestinal bifurcation, near oral sucker. Two lobed testes, posterior and extracecal in position: external vesicula seminalis well developed. Cirrus sac elongate with internal vesicula seminalis; cirrus unarmed. Ovary median, between the testes, immediately posterior to Mehlis' gland; receptaculum absent. Uterus with ascending limb only, forming intracecal transverse slings. Metraterm long, with thickened walls and opening at the genital pore. Vitellaria follicular, in two lateral extracecal bands, running forward from anterior border of testes. Excretory pore dorsal and posterior, receiving the two main excretory vessels. Eggs numerous, small and operculate with long polar filaments. Adults in intestinal ceca and bursa Fabricius of birds. Genotype: *U. gippyensis* n. sp."

The genus was included in the subfamily Notocotylinae, family Notocotylidae, and distinguished from other genera: *Notocotylus* Diesing, 1839; *Catatropis* Odhner, 1905; *Paramonostomum* Lühe, 1909; *Quinqueserialis* Skvorzov, 1934; *Hofmonostomum* Harwood, 1939; and *Tristriata* Belopolskaia, 1953

MATERIAL AND METHODS

During the summer months of 1963, 1964, 1965 and 1966, over 5000 specimens of *Hydrobia salsa* were examined for infection by larval trematodes. The snails were identified by Dr. W. K. Emerson of the American Museum, New York. This is a somewhat rare, prosobranchiate species, described by Pilsbry (1905) as

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[1] Investigation supported by Grant NSF-GB-3606, continuation of G-23561.

Paludestrina salsa. During the summers of 1963 and 1964, these snails were common in Nobska Pond near Woods Hole, Massachusetts, and most of the specimens were taken from an area near the connection of the pond with Vineyard Sound. In the fall of 1964, the pond was "treated" and most of the invertebrates, including snails, were killed. In the summer of 1965, *H. salsa* was found in nearby Oyster Pond and the study was continued. The results have been rewarding; ten different species of larval trematodes have been recognized; five of them are notocotylid cercariae. The methods and procedures employed have been described in earlier reports (Stunkard, 1960, 1966a, 1966b, 1967) on the morphology and life-cycles of notocotylid species. In the (1966a) paper, the writer reported that the five notocotylid cercariae included representatives of all three larval types dis-

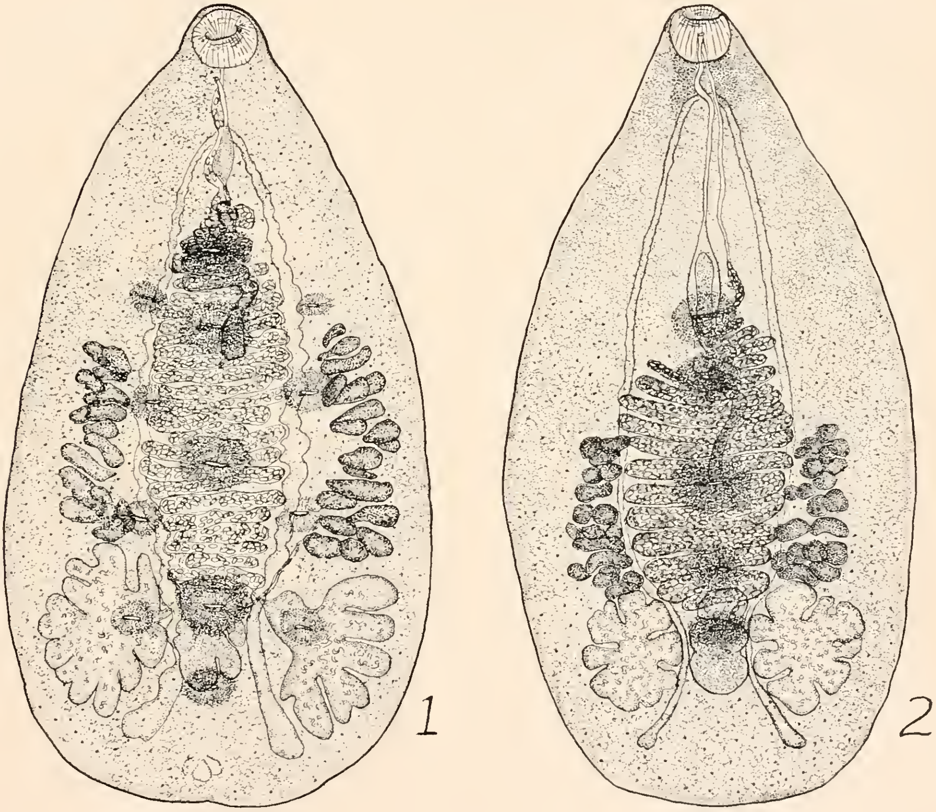
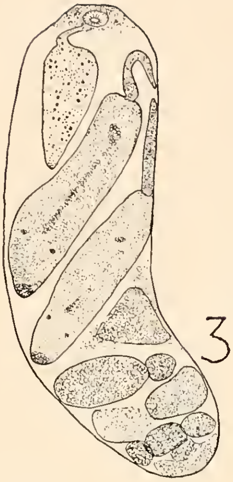


Plate I

FIGURE 1. *Uniserialis breviserialis*, adult specimen, ventral view, somewhat flattened, fixed and stained, 2.3 mm. long, from the bursa of a domestic duckling, 12 days after metacercaria was fed.

FIGURE 2. *Uniserialis gippyensis* Beverley-Burton, 1958, paratype specimen, ventral view, 1.62 mm. long, from bursa of *Anas platyrhyncha*. The ventral glands are not visible in this specimen; their size and location on other worms were determined and added in the drawing.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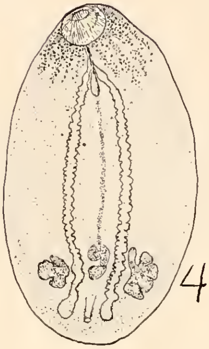
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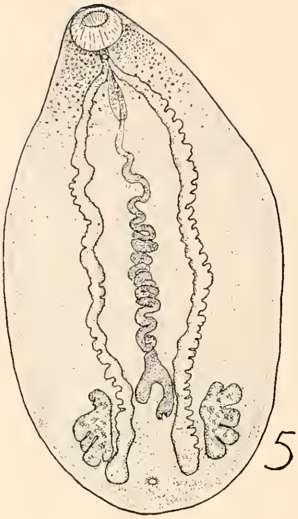
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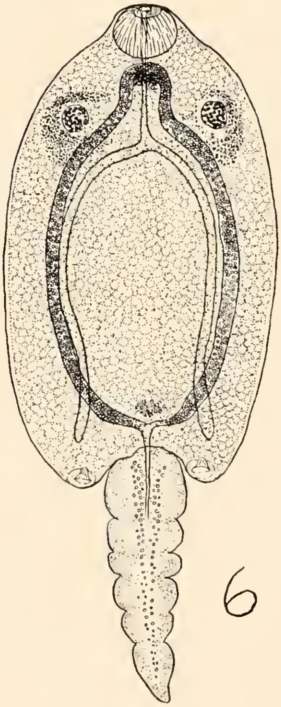
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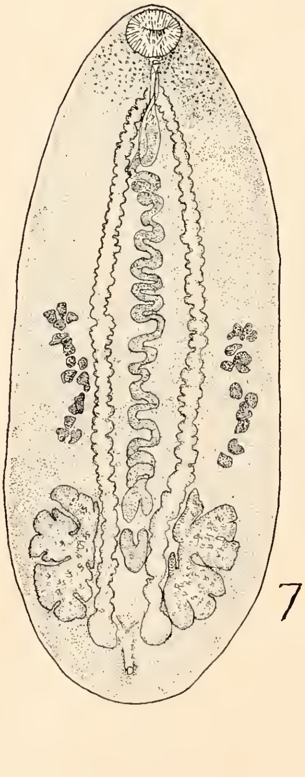
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Plate II

tinguished by Rothschild (1938) on differences in the structure of the excretory system and designated as the Yenchingensis, the Monostomi, and the Imbricata Groups. Two of the cercariae belong to the Yenchingensis Group; they develop to maturity in the digestive caeca of ducklings and were described by Stunkard (1966b) as *Nototylus minutus* Stunkard and Dunihue, 1931 and *Notocotylus atlanticus sp. nov.* Two cercariae belong to the Monostomi Group; they develop in the lumen of the intestine of chicks and ducklings and were described by Stunkard (1967) as *Paramonostomum alveatum* (Mehlis in Creplin, 1846) and *Paramonostomum parvum* Stunkard and Dunihue, 1931. The fifth cercarial species belongs to the Imbricata Group; these larvae develop in the bursa Fabricius of chicks and ducklings, and form the subject of the present report.

These worms are similar in many respects to those from the intestinal caeca and bursae Fabricii of mallard ducks, described by Beverley-Burton (1958) as *Uniserialis gippyensis*; compare Figures 1 and 2. According to Beverley-Burton, the worms described as *U. gippyensis* have only one row of ventral glands, median in position and five in number. The present specimens have the five median glands and, in addition, four small lateral glands on either side, situated in the intervals between the median glands. These small lateral glands are recognizable in most living specimens but are rarely visible in fixed and stained preparations. Indeed, the large, median glands can not be seen in many whole-mount individuals. It appeared possible that the present worms are congeneric with those described by Beverley-Burton and in an attempt to resolve the problem, the specimens of *U. gippyensis* deposited in the British Museum (Natural History) were borrowed through the kindness of Mr. Stephen Prudhoe. The material in the British Museum consists of one slide on which there are three specimens, all lightly stained. The largest and apparently the most representative specimen, ringed on the coverglass, is reproduced (Fig. 2); it is 1.62 mm. long; 0.81 mm. wide; oral sucker, 0.14 mm. in diameter; distance from anterior end to base of cirrus sac, 0.66 mm.; testes measure 0.25 by 0.19 mm.; ovary, 0.13 mm. in diameter; vitellaria extend 0.32 mm. and are situated in the posterior half of the body. In this specimen the median glands are not recognizable although they can be seen on the other two worms. The other worms are slightly smaller but very similar, and all agree completely with the specific description as given by Beverley-Burton. No lateral glands are visible on any of the specimens, but the staining is faint and it is probable that if present, they would not be recognizable.

The Imbricata cercariae are the largest of the notocotylid larvae found in *H. salsa*. They emerge principally after 11 AM and some were swimming at 4 PM, but most of them encysted after swimming for an hour or two. Feeding of encysted metacercariae to chicks and domestic Pekin ducklings yielded developing and

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FIGURE 3. *U. breviserialis*, redia, natural infection, fixed under a coverglass, 0.87 mm. long.

FIGURE 4. *U. breviserialis*, juvenile specimen, flattened under a coverglass, stained and mounted, 0.75 mm. long, four days in domestic duckling.

FIGURE 5. *U. breviserialis*, juvenile specimen, flattened under a coverglass, stained and mounted, 1.12 mm. long, six days in a domestic duckling.

FIGURE 6. *U. breviserialis*, young cercaria, from fixed and stained specimen, flattened under a coverglass, with details added from sketches of living specimens.

FIGURE 7. *U. breviserialis*, juvenile specimen, flattened under a coverglass, stained and mounted, 1.56 mm. long, nine days in a domestic duckling.

gravid worms, all of which were taken from the bursa Fabricius. The worms adhere tenaciously to the wall of the bursa and when removed, the caeca are bright red with blood from the host. The corpuscles are clearly visible in fixed and stained worms. Both median and lateral ventral glands are visible in juvenile worms, six days in a chick, as well as in sexually mature specimens. A series of drawings (Figs. 4, 5 and 7) shows development of worms from four to nine days in the bursa of domestic ducklings. The smallest sexually mature worm, fixed without pressure, is 1.19 mm. long and 0.65 mm. wide; the vitellaria extend through a distance of 0.24 mm.; the testes measure 0.18 by 0.16 mm.; the cirrus sac is 0.19 mm. long and 0.055 mm. wide; the ovary is 0.16 mm. long and 0.072 mm. wide; Mehlis' gland is 0.080 mm. wide and 0.060 mm. long. The metacercariae were fed August 14, 1965, and the bird was autopsied August 30, 1965.

DESCRIPTIONS

Adult (Fig. 1)

The body is ovate, much flattened, more pointed anteriorly and rounded posteriorly. It is convex dorsally, concave ventrally, with the edges of the body turned ventrad and mediad. Fixed and stained specimens measure 1.19 to 2.54 mm. in length and 0.56 to 1.66 mm. in width. The cuticula is thin; fine spines were observed on the ventral surface of living worms but they do not show on fixed and stained specimens. The body wall is delicate, the musculature is weak; the longitudinal muscles are best developed. The ventral surface bears five median, protrusible glands that are conspicuous in living specimens. They are circular to oval in outline with transverse slit-like openings. The anterior and posterior glands measure 0.12 to 0.15 mm. in diameter; the three middle ones are somewhat larger and measure 0.16 to 0.20 mm. in diameter. The most anterior gland is situated about its diameter posterior to the base of the cirrus sac; the second gland is at the level of the anterior ends of the vitellaria; the third gland is near the middle of the vitelline zone; the fourth gland is at or slightly posterior to the level of the caudal ends of the vitellaria; and the most posterior gland is at the ovarian level. The lateral glands are smaller than the median ones; they measure 0.09 to 0.12 mm. in diameter, and are situated in the intervals between the median ones. The three anterior ones are in the fields of the digestive caeca and the most posterior glands are ventral to the antero-median lobes of the testes.

The excretory pore is dorsal, about midway between the ovary and the posterior end of the body. The bladder is small and the collecting ducts extend forward, forming a loop that crosses the body anterior to the cerebral ganglia. Dendritic branches from the longitudinal ducts form a complex network of excretory channels.

The oral sucker is terminal, 0.13 to 0.20 mm. in diameter; the mouth is slightly ventral; the esophagus is short, about the length of the sucker; the caeca extend posteriad, lateral to the uterine loops, turn mediad to pass between the testes and ovary, and end blindly posterior to the testes.

The testes are situated in the extracaecal areas near the posterior end of the body. They are deeply lobed, and vary in size from 0.18 by 0.16 mm. to 0.50 by 0.375 mm. Sperm ducts arise at the antero-median faces and unite a short distance anterior to Mehlis' gland to form the vas deferens which passes anteriad, dorsal to the uterus. At about the level of the anterior ends of the vitellaria, it expands to form a coiled external seminal vesicle which continues the forward course to the cirrus sac. A coiled internal seminal vesicle occupies the posterior third to one-half of the cirrus sac and is continued by the ejaculatory duct; both are enclosed in prostatic cells. The cirrus sac leads to the genital pore, located anterior to the cerebral ganglia and at or near the level of the posterior border of the oral sucker. The cirrus sac is dorsal to the metraterm and measures from 0.19 to 0.40 mm. in length and 0.055 to 0.15 mm. in greatest width. It is located in the anterior fourth of the body.

The ovary is lobed, usually longer in the antero-posterior axis and increases in size as the worm matures. In a young specimen it may be 0.16 by 0.072 mm. and in a large, fully mature worm it may be 0.28 by 0.20 mm. The oviduct arises at the antero-dorsal margin and receives a common vitelline duct as it enters Mehlis' gland, which is somewhat smaller and immediately anterior to the ovary. There is no seminal receptacle and the initial coils of the uterus are filled with spermatozoa. The uterus passes forward in intercaecal, transverse loops, 15 to 25 in number, to communicate with the metraterm. The metraterm is somewhat shorter than the cirrus sac, is ventral in position, has a weak muscular wall, and opens at the genital pore posterior to the opening of the cirrus sac. The vitellaria consist of 15–20 discrete, irregularly shaped follicles which occupy the extracaecal areas from the testes to the level of the external seminal vesicle. They extend through a distance of 0.22 to 0.62 mm. and in large part are situated in the middle third of the body. Collecting ducts course posteriad along their median faces and at the posterior end of the vitellaria pass mediad, ventral to the caeca, then turn dorsad, joining above Mehlis' gland to form a vitelline receptacle from which the short common duct leads to the oviduct. The eggs are operculate, 0.019 to 0.020 mm. long, 0.011 to 0.013 mm. wide, provided with long polar filaments, and embryonated when passed.

Redia (Fig. 3)

The rediae are oval to sausage-shaped to elongate; extended, they are cylindrical with conical posterior ends. Small rediae are colorless, actively motile but without feet, and one which measured 0.10 mm. in length contained a small daughter as well as germ balls of developing cercariae. Locomotion is accomplished by contraction of the circular and logitudinal muscles of the body wall. As the rediae grow and become filled with progeny, movement is less and less active. Older rediae have orange-yellow droplets, 0.002 to 0.006 mm. in diameter, in the body wall; the largest extend to a length of 1.00 mm.; the specimen shown in Figure 3, fixed and stained, is 0.87 mm. long and 0.23 mm. wide. In the older rediae the pharynx measures 0.042 to 0.052 mm. in diameter, the esophagus is about the same length, and the intestine, which extends to the middle of the body in young rediae, is restricted to the anterior third or fourth of the body length. The birth pore is ventral at the level of the esophagus. There are two excretory pores, one on either side near the middle of the posterior half of the body. From each pore a duct passes forward, just past the middle of the body where it divides into anterior and posterior branches. Each branch terminates in a flame-cell, one at the level of the esophagus, the other posterior to the excretory pore.

Cercaria (Fig. 6)

The cercariae are large; they emerge from the rediae in very immature condition, about one-half the size they eventually attain. On emergence from the redia into the haemal sinus of the snail, the tail is so small and weak that the cercaria cannot swim if liberated by crushing the snail. Figure 3 is made from sketches of a young specimen. Alive, it extended to a length of 0.30 mm., fixed and stained it is 0.20 mm. long and 0.12 mm. wide. In it the excretory ring is complete but the common stem extends into the tail and the excretory pores are situated on the sides of the tail. The excretory system develops in the manner described for Imbricata cercariae by Rothschild (1938: Figs. 30, 31, 32, 34, 38), with the primary collecting ducts fusing anteriorly to form the loop that extends across the body anterior to the cerebral ganglia and the median eye-spot. As the cercaria matures in the haemal sinuses of the snail, the ring becomes filled with the concretions 0.003–0.005 mm. in diameter and the portion of the excretory system in the tail atrophies as a new definitive excretory pore develops from the dorsal wall of the expanding excretory bladder. The study of the flame-cell pattern has been disappointing. It is probable that the formula is $2[(3+3+3)+(3+3+3)]$, as reported by Martin (1956) and Odening (1966) for other notocotylid cercariae. In young cercariae the formula is $2[(1+1+1)+(1+1+1)]$, and in older cercariae the anterior and posterior groups each have three cells, but the cystogenous cells fill so early that not all the flame-cells and capillaries have been observed in the mid-region of the body. As noted, the cystogenous cells fill the parenchyma and obscure other structures; the secretion appears in the form of short, bacilliform rods. Normally emerged cercariae vary from 0.30 to 0.60 mm. in length and 0.14 to 0.25 mm. in width. The postero-lateral ends of the body bear eversible, retractile locomotor appendages which function in creeping movements of the body. When the body is extended, they are close together, separated only by the base of the tail which is between and ventral to them; as the body contracts they separate and serve as fulcra for the next extension of the body. The tail is simple, slender, 0.04 to 0.08 mm. in width at the base, and about the same length as the body. When either is contracted, the other is elongated. The wall of the tail is composed of external circular and internal longitudinal muscles which enclose loose parenchymal tissue. In swimming, the body is contracted, bent ventrally, almost circular, while the tail is extended and lashes vigorously. The ocelli are formed while the cercariae are in the rediae (Fig. 3), and increase to a diameter of 0.016 to 0.024 mm.; they are provided with lenses and are connected by short nerves to the cerebral ganglia. The median eye-spot, usually lacking in young cercariae, often becomes well organized as a dark ring in emerged individuals. Diffuse, dendritic strands of pigment surround the ocelli, permeate the anterior end of the body and extend posteriorly, especially along the digestive caeca. The oral sucker measures 0.04 to 0.05 mm. in diameter, the esophagus is about the same length; it passes backward below the commissure of the nervous system and above the excretory ring. Immediately behind the level of the cerebral ganglia it communicates with the intestinal caeca. The caeca extend posteriad, dorsal and medial to the excretory ring, which they cross near the posterior end of the body to terminate in the extracaecal areas. Deeply staining germinal cells, situated immediately anterior to the caudal end of the excretory ring, are the primordia of the gonads, and a strand of these cells extends anteriorly in the median plane.

Metacercaria

Infected snails were identified by isolation. The cercariae begin to emerge about 11:00 AM and swim toward the light side of the container. By 3:00 PM, almost all are encysted, on the shell of the snail from which they emerged, the wall of the container, or on strands of algae. The cysts are the largest of the five notocotylid species and average measurements are 0.195 mm. in external and 0.175 mm. internal diameter. The worms do not develop in their cysts; they are infective immediately and become sexually mature in about two weeks in the bursae Fabricii of ducklings and chicks.

Discussion

The present specimens are very similar, morphologically, to *Uniserialis gippyensis* Beverley-Burton, 1958. They are from the same site, the bursa Fabricius of birds, and from the same or related host species. The principal difference is the presence on the ventral surface of lateral glands which were not described for *U. gippyensis*. But these glands are rarely visible in fixed and stained specimens. If they do occur in *U. gippyensis*, the present specimens are obviously congeneric with those of Beverley-Burton, and on that presumption, they are described as a new species, *Uniserialis breviserialis*. Type and paratype specimens are deposited in the Helminthological Collection of the U. S. National Museum under the numbers 61,186 and 61,187. Specific differences between *U. gippyensis* and *U. breviserialis* are recognizable in the length of the cirrus sac and the location of the gonads and vitellaria. In *U. gippyensis* the cirrus sac is about twice as long; it extends one-third of the length of the body; in *U. breviserialis* it is short, less than one-fourth of the body length. In *U. gippyensis* the reproductive organs are more posteriorly situated; the vitellaria are in the posterior half of the body whereas in *U. breviserialis* the vitellaria are situated largely in the middle third of the body.

The validity of the genus *Uniserialis* is questionable. Baer and Joyeux (1961) suppressed *Uniserialis* as identical with *Notocotylus* Diesing, 1839 and the presence of lateral glands on the ventral surface of the present specimens seemingly supports that action. But there are other considerations which may validate the genus *Uniserialis*. Miriam Rothschild (1938) recognized three types of notocotylid cercariae, designated the Yenchingensis, the Monostomi, and the Imbricata Groups, respectively, based on the structure of the excretory system. Stunkard (1966a) found that *Notocotylus minutus* and *Notocotylus atlanticus* have Yenchingensis-type cercariae and develop in the intestinal caeca; that *Paramonostomum alveatum* and *Paramonostomum parvum* have Monostomi-type cercariae and develop in the lumen of the intestine; whereas the present species, *Uniserialis breviserialis*, has Imbricata-type cercariae and localizes in the bursa Fabricius. The apparent correlation of cercarial type, generic allocation and sites of infection is disturbed by the report of Rothschild (1941) that two species of Yenchingensis-type cercariae developed in the intestinal caeca of ducks into flukes of the genus *Paramonostomum*. Furthermore, Odening (1966) reported that five species of *Notocotylus*: *N. pacifer* (Noble, 1933); *N. ephemera* (Nitzsch, 1807); *N. noyeri* Joyeux, 1922; *N. regis*

Harwood, 1939; and *N. ralli* Baylis, 1936, have Monostomi-type cercariae, whereas *Catatropis verrucosa* (Fröhlich, 1789) has Imbricata-type cercariae.

Odening stated that the cercariae of *C. verrucosa* lack eye-spots, have short, stumpy tails, and encyst in the snails in which they are produced, *viz.*, *Segmentina nitida* (O.F.M.) and *Gryaulus albus* (O.F.M.). The adults were raised in ducklings. The life-cycle of *C. verrucosa* as given by Odening recalls the account of Joyeux (1922) who reported that stumpy-tailed cercariae without eye-spots from *Planorbis rotundatus* Poiret developed in ducklings to adults which were identified as *Notocotylus attenuatus*. The adults were not described but Dubois (1951) examined specimens deposited in the Zoological Institute of the University of Neuchâtel and declared that the worms were not *N. attenuatus* but *C. verrucosa*. Szidat (1930) had reported that *Cercaria ephemera* Nitzsch, 1807 from *Planorbis corneus* is the larva of *C. verrucosa*, but L. and U. Szidat (1933) assigned the adults to a new species, *Notocotylus thienemanni*. Erkina (in Skrjabin *et al.*, 1963) described a large cercaria from *Bithynia tentaculata* and *Bithynia leachi* with three eye-spots and a long tail as the larva of *Catatropis verrucosa*. Martin (1956) described *Catatropis johnstoni* n. sp., and its life-cycle. The larvae were found in the prosobranch snail, *Cerithidea california;* they had long tails, eye-spots, and belonged to the Imbricata-group of cercariae.

Discussing the reports of Erkina and Martin, Odening (1966: 229) stated, "Es scheint kaum möglich, dass ein und dieselbe Art zwei ganz verschiedene Larventypen hat; folglich kann es sich wohl nur bei einem der beiden Zyklen um den von *C. verrucosa* handeln. Ob es sich nun hierbei um zwei Arten handelt, die als Adulti kaum oder nicht unterscheidbar sind, kann vorerst nicht entschieden werden. . . . Es erhebt sich die Frage, ob nicht jener merkwürdige, von Joyeux entdeckte Typ monostomer Cercarien für die Gattung *Catatropis* characteristisch wäre. Diese Frage lässt sich in Anbetracht der Differenz zwischen den Angaben von Erkina und den hier geschilderten Ergebnissen (sowie denen von Joyeux) über *Catatropis verrucosa* nicht beanworten. Leider liegen keine Angaben über Entwicklung anderer *Catatropis*-Arten vor, bis auf die Resultate von Martin (1956) über '*Catatropis johnstoni* Martin, 1956.' Die Zugehörigkeit dieser Art zur Gattung *Catatropis* ist jedoch fraglich, denn es heisst in der Diagnose: 'Median ventral glandular ridge from ovarian to mid-cirrus level. Lateral ventral glands lacking.'

"Diese Art würde in dem gleichen Verhältnis zur Gattung *Catatropis* stehen wie die Gattung *Uniserialis* Beverley-Burton, 1958, zur Gattung *Notocotylus*. Andererseits ist der von Martin beschriebene mediane Drüsenkiel aus einzelnen querovalen Drüsen zusammengesetzt. Die Cercarie von '*Catatropis johnstoni*' gehört zur 'Imbricata'-Gruppe (Rothschild), hat einen langen Schwanz und drei Augen. Die zugehörigen Redien schmarotzen bei einem Prosobranchier, der an der californischen Küste lebt. Die Gruppenzugehörigkeit der von Erkina für *C. verrucosa* beschriebenen Cercarie ist nicht eindeutig erkennbar; aus den Zeichnungen könnte man vielleicht entnehmen, dass es sich um eine Cercarie der 'Yenchingensis'-Gruppe handelt (vgl. auch Ševcov und Zaskind, 1960). Der von Joyeux entdeckte stummelschwanzige und augenlos Type monostomer Cercarien wurde auch bei *Parapronocephalum symmetricum* Belopol'skaja, 1952, nachgewissen (s. Skrjabin *et al.*, 1955). Die Redien schmarotzen in Meeresprosobranchiern. Die Cercarie gehört zur 'Monostomi'-oder zur 'Imbricata'-Gruppe (die Gruppenzugehörigkeit wurde nicht angegeben, es kann nur die 'Yenchingensis'-Gruppe ausgeschlossen werden)."

Uniserialis breviserialis, like species of *Catatropis,* has Imbricata-type cercariae. The significance of groups of notocotylid cercariae, their generic allocations and infective sites are yet dubious, and it is apparent that discrimination and discretion will be required for a solution of the taxonomic problems in the family Notocotylidae.

SUMMARY

Imbricata-type cercariae from *Hydrobia salsa,* a brackish-water, prosobranch snail taken near Woods Hole, Massachusetts, emerge shortly before noon, are photopositive and encyst after swimming for a few minutes to three to four hours. Metacercariae were fed to chicks and domestic ducklings and developed to mature worms after about two weeks in the bursae Fabricii of these birds. Adult and larval stages are described and figured. The worms belong in the family Notocotylidae and are assigned to the genus, *Uniserialis* Beverley-Burton, 1958. Systematic problems of genera in the family are discussed.

LITERATURE CITED

BAER, J. G., AND CH. JOYEUX, 1961. Classe des Trématodes. *In:* Traité de Zoologie, P. P. Grassé, **4**: 561–692.

BEVERLEY-BURTON, MARY, 1958. A new notocotylid trematode, *Uniserialis gippyensis* gen. et sp. nov., from the mallard, *Anas platyrhyncha platyrhyncha* L. *J. Parasitol.,* **44**: 412–415.

DUBOIS, G., 1951. Étude des trématodes Nord-Americains de la collection E. L. Schiller et revision du genre *Notocotylus* Diesing, 1839. *Bull. Soc. Neuchât. Sci. Nat.,* **74**: 41–76.

JOYEUX, CH., 1922. Recherches sur les Notocotyles. *Bull. Soc. Path. Exot.,* **15**: 331–343.

MARTIN, W. E., 1956. The life cycle of *Catatropis johnstoni* n. sp. (Trematoda: Notocotylidae). *Trans. Amer. Micros. Soc.,* **75**: 117–128.

ODENING, K., 1966. Physidae und Planorbidae als Wirte in den Lebenszyklen einheimischer Notocotylidae (Trematoda: Paramphistomidea). *Zeitschr. Parasitenk.,* **27**: 210–239.

PILSBRY, H. A., 1905. A new brackish-water snail from New England. *Nautilus,* **19**: 90–91.

ROTHSCHILD, MIRIAM, 1938. Notes on the classification of cercariae of the superfamily Notocotyloidea (Trematoda), with special reference to the excretory system. *Novit. Zool.,* **41**: 75–83.

ROTHSCHILD, MIRIAM, 1941. Note on the life histories of the genus *Paramonostomum* Lühe, 1909 (Trematoda: Notocotylidae) with special reference to the excretory vesicle. *J. Parasitol.,* **27**: 363–365.

SKRJABIN, K. I., 1953. Trematoden der Tiere und des Menschen. Grundlagen der Trematodologie (Russich), vol. 8. (Cited after Odening, 1966.)

SKRJABIN, K. I., N. P. SICHOBALOVA, A. M. PETROV AND M. M. LEVASOV, 1963. Der Aufbau der helminthologischen Wissenschaft und Praxis in the UdSSR. Bd. 2. (Russich). Verl. Akad. Wiss. UdSSR. (Cited after Odening, 1966.)

STUNKARD, H. W., 1960. Studies on the morphology and life-history of *Notocotylus minutus* n. sp., a digenetic trematode from ducks. *J. Parasitol.,* **46**: 803–809.

STUNKARD, H. W., 1966a. Further studies on digenetic trematodes of the family Notocotylidae. *Biol. Bull.,* **131**: 409.

STUNKARD, H. W., 1966b. The morphology and life-history of *Notocotylus atlanticus* n. sp., a digenetic trematode of eider ducks, *Somateria mollissima,* and the designation, *Notocotylus duboisi* nom. nov., for *Notocotylus imbricatus* (Looss, 1893) Szidat, 1935. *Biol. Bull.,* **131**: 501–515.

Stunkard, H. W., 1967. Studies on the trematode genus, *Paramonostomum* Lühe, 1909 (Digenea: Notocotylidae). *Biol. Bull.*, **132**: 133–145.

Stunkard, H. W., and F. W. Dunihue, 1931. Notes on trematodes from a Long Island duck with description of a new species. *Biol Bull.*, **60**: 179–186.

Szidat, L., 1930. Die Parasiten des Hausgeflügels. 4. *Notocotylus* Diesing und *Catatropis* Odhner, zwei, die Blinddärme des Geflügels bewohnende monostome Trematodengattungen, ihre Entwicklung und Uebertragung. *Arch. f. Geflügelk.*, **4**: 105–114.

Szidat, L., and Ursula Szidat, 1933. Beiträge zur Kenntnis der Trematoden der Monostomidengattung *Notocotylus* Dies. *Zentrb. Bakt., Abt. I*, **129**: 411–422.

THE GROWTH AND ACTIVITY OF THE CORPORA ALLATA IN THE LARVAL FIREBRAT, THERMOBIA DOMESTICA (PACKARD) (THYSANURA, LEPISMATIDAE)

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Although the post-embryonic development of the Apterygota (including the Archaeognatha and Thysanura) is essentially continuous and progressive, it shows some abrupt changes similar to those associated with the metamorphoses of higher insects. In particular, the integument of newly hatched larvae lacks scales, although it bears setae; if scales develop, they do so either at the second molt, as in Archaeognatha, or at the third, as in most lepismatid Thysanura (Delany, 1957).

The processes of scale and bristle formation in Thysanura resemble those found in the higher insects (Schmidt, 1959; Richter, 1962). Furthermore the development of scales in *Lepisma* resembles metamorphic changes in that it appears to depend on the hormonal system of the insect (Piepho and Richter, 1959; Richter, 1962). Thus fragments of the integument from newly hatched *Lepisma*, if implanted into adults, molt when the adult molts and develop scales, suggesting that the control is humoral. However, the time that elapses between hatching and ecydsis to the fourth larval stage in *Lepisma* averages 17.8 days, as opposed to approximately 43 days for each stadium in the adult (Sweetman, 1952), so that in Piepho and Richter's experiments, the epidermal cells could have had time to differentiate independently between implantation and the subsequent adult ecdysis.

Other recent, experimental studies on the hormonal control of molting and reproduction in lepismatids have emphasized that the endocrine systems of these insects are similar in structure to those of the winged insects and that, at least with respect to the initiation of molting and the deposition of yolk, they appear to function in the same way (Yashika, 1960; Watson, 1963a, 1964a, 1965 and unpublished results; Rohdendorf, 1966).

The question therefore arises: If the development of scales in Thysanura is not a spontaneous event, is it regulated by the corpus allatum? In other words, do these ametabolous insects show an early stage in utilization of the allatal secretion as a morphogenetic agent?

The present paper describes experiments to check for the existence of humoral control over scale formation in lepismatids, and examines the physiological activity of the corpora allata during the life of the firebrat. Brief notes on the work have already appeared (Watson, 1936b, 1965). Attempts to influence scale formation in early larvae and in regenerating integument have so far yielded inconclusive results and will not be documented here.

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[1] Much of the work described in this paper was carried out in the Developmental Biology Center, Western Reserve University, Cleveland, Ohio.

MATERIALS AND METHODS

1. *Experimental animals*

All experiments were performed on the firebrat, *Thermobia domestica* (Packard). Larvae and adults were reared under optimal conditions, at 37° C. and 84% relative humidity, as described in Watson (1964a).

2. *Implantations*

Implantations were carried out much as described by Piepho and Richter (1959). First stage larvae less than three hours old were anesthetized with carbon dioxide. The terminalia were removed from the abdomen and the insect was then cut across at the junction of the thorax and abdomen. The abdominal fragment was placed under 0.9% saline until implantation. An adult firebrat three to five days after ecdysis, and presumed to be on the point of initiating a molt (*cf.* Watson, 1964a), was anesthetized for one or two minutes with carbon dioxide, the abdomen was descaled and the larval fragment implanted through a slit in the third abdominal tergum; the wound was sealed with a paraffin-beeswax mixture. The adult was returned to 37° C. and 84% relative humidity.

The recipients of implants were fixed in neutral formalin at various times after implantation, and were sectioned serially for examination of the larval fragment.

3. *Histological measurments*

Studies of the corpora allata of other insects have indicated that changes in the physiological activity of the gland may involve changes in the volumes of cytoplasm and nucleus, commonly expressed as a ratio between cytoplasm and nucleus, with or without changes in the number of cells (*cf.* Pflugfelder, 1958; Scharrer, 1964). In the case of the firebrat, however, Watson (unpublished data and below) has shown that neither the nuclear diameter nor the number of cells alters during short-term fluctuations in the size of the corpora allata, fluctuations that are correlated with changes in the physiological activity of the gland.

The volume of cytoplasm in each allatal cell is therefore an appropriate measure of physiological activity; and as nuclear volume is constant, total cell volume is an equally valid index.

Firebrats in which such histological measurements were to be made were fixed in neutral formalin and sectioned serially at 4–8 μ.

(a) Numbers of allatal nuclei

Nuclei and fragments were counted in all sections of the corpora allata. The resulting numbers were corrected for fragmentation by the formulae of Marrable (1962).

(b) Nuclear diameter

The major and minor axes of the almost spherical nuclei were measured in 10 allatal cells in each of a series of 24 firebrats of various ages. An average diameter was calculated for each animal, and the individual means were averaged over the sample. The resulting average, 6.323 μ (S.E. = 0.067 μ), was used for all later calculations involving nuclear volumes.

(c) Volume of the corpora allata

Volumes were measured by summing the areas of all the sections of the corpora allata, using a squared eyepiece micrometer, and multiplying by the thickness of the section.

All other cytological statistics used in this paper were calculated from the above three measurements.

4. *The assay of allatal activity*

The juvenilizing activity of the corpora allata was assayed by a modification of the Polyphemus test (Gilbert and Schneiderman, 1960). Chilled pupae of the saturniid *Antheraea polyphemus* (Cram.) were placed at 25° C., until the first signs of epidermal retraction from the facial window indicated the beginning of adult development. Maxillae (which contain the corpora allata) or the ventral halves of heads sufficient to provide approximately 100 allatal cells (see below) were implanted into the midrib of the pupal antenna. Dorsal halves of the head, or an approximately equal volume of body tissue, served as control in the other antenna, and in later comparative experiments corpora allata from two different stages were implanted into the two antennae. The antennae of the resulting moth were scored for pupal characters on the scale of Gilbert and Schneiderman (1960). As the critical period for the action of the juvenile hormone ends shortly after retraction of the facial epidermis, the juvenilizing effect of the implant is a reflection of its secretory state at or immediately after the time of implantation.

5. *Analyses of data*

Statistical techniques referred to below are described in Siegel (1956) or Bailey (1959).

RESULTS

1. *Timetable of development*

At 37° C. and 84% R.H. growth from hatching to ecdysis to the fourth larval stage, when the scales first appear, occupies an average of 8.5 days; the mean lengths of the first three stadia, with standard errors, are 24.9 ± 0.47 hours, 69.9 ± 0.49 hours and 109.1 ± 5.14 hours. The larvae commence feeding in the third stadium, the residual yolk then being exhausted.

The sequence of epidermal events during the third stadium appears in Figure 1. At 18 hours after ecdysis, the epidermis appears inactive; mitosis follows, in most cases some time between the 18th and 48th hour, so that by the 60th hour, pycnotic epidermal nuclei are present in all larvae. Extensive RNA synthesis, as reflected by the basophilia of the trichogen and presumptive scale-forming cells, generally commences between the 18th and 48th hours, but strong basophilia may not develop until the third day. The epidermis starts to retract between 60 and 72 hours, the first scale-forming processes appear by 84 hours, and ecdysis follows approximately 24 hours later.

Thus any mechanisms influencing scale formation must act before the 48th hour after ecdysis, by which time the presumptive scale-forming cells are becoming recognizable.

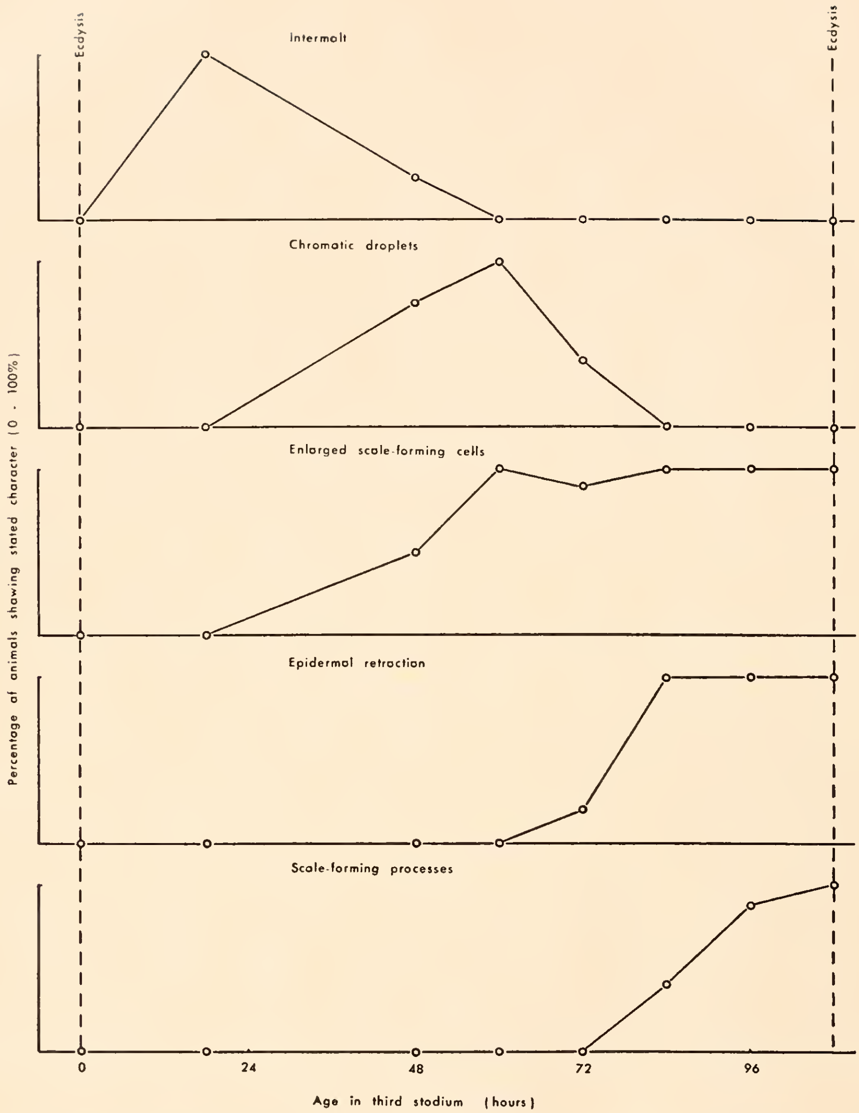

FIGURE 1. Timetable of epidermal events during the 3rd stadium of *Thermobia*.

2. *Implantation experiments*

The experiments on implantation unequivocally confirmed the interpretations of Piepho and Richter (1959). The implants had molted and developed scales when the adult recipients had molted, five days or more after implantation; and in a few cases, scales had formed within 48 hours of implantation (Fig. 2). Clearly, scale formation it not simply a spontaneous, time-dependent differentiation; the potential for it is present in first stage larvae, and can be realized immediately in an appropriate environment. As some of the implants which had developed scales still contained abundant yolk in close proximity to the integument, it seems unlikely that a component of yolk directly inhibits scale formation. The "appropriate" environment may therefore be one which differs hormonally from that of the early larvae, as Piepho and Richter (1959) have suggested.

Could such a difference involve the juvenile hormone? In the present study, the histophysiological aspects of this question have been considered, particularly the pattern of growth in the corpus allatum and the secretory activity of the gland during larval and adult life.

3. *The post-embryonic growth of the corpus allatum*

(a) The number of cells in the corpus allatum

The number of cells in each corpus allatum increases as the firebrat grows. Watson (1963a), working with data not corrected for fragmentation, described linear growth in the adult corpus allatum, and it now appears that the linear relationship extends through the larval stages (Fig. 3). The fitted regression in Figure

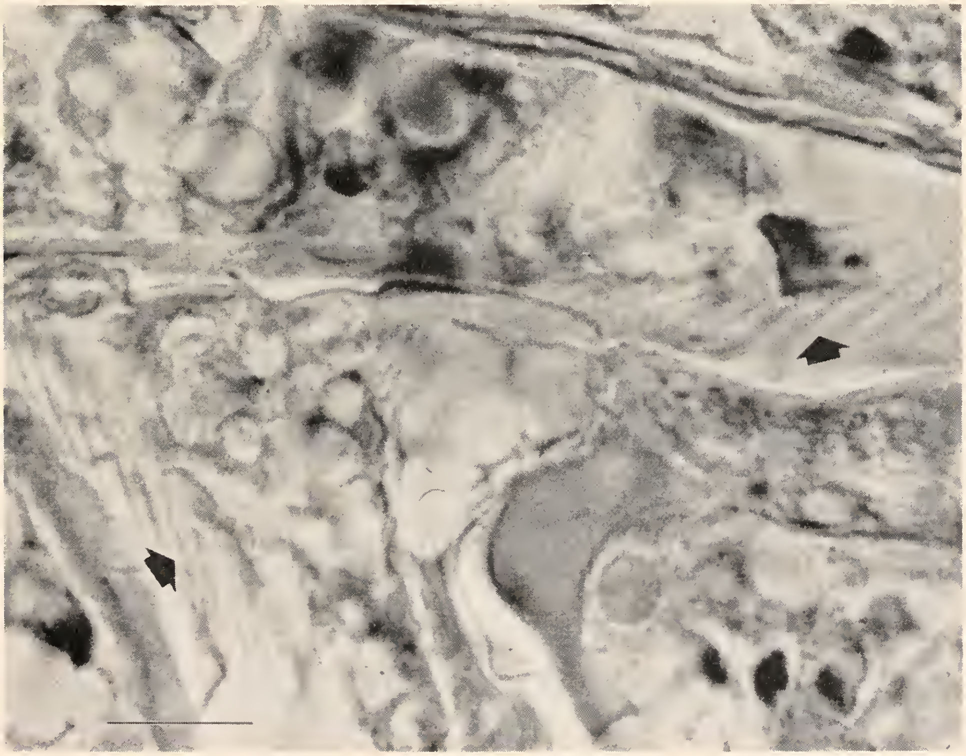

FIGURE 2. Section of integument from *Thermobia* hatchling implanted into molting adult, and left for 48 hours. The prematurely-formed scales are indicated by arrows; the marker represents 10 μ.

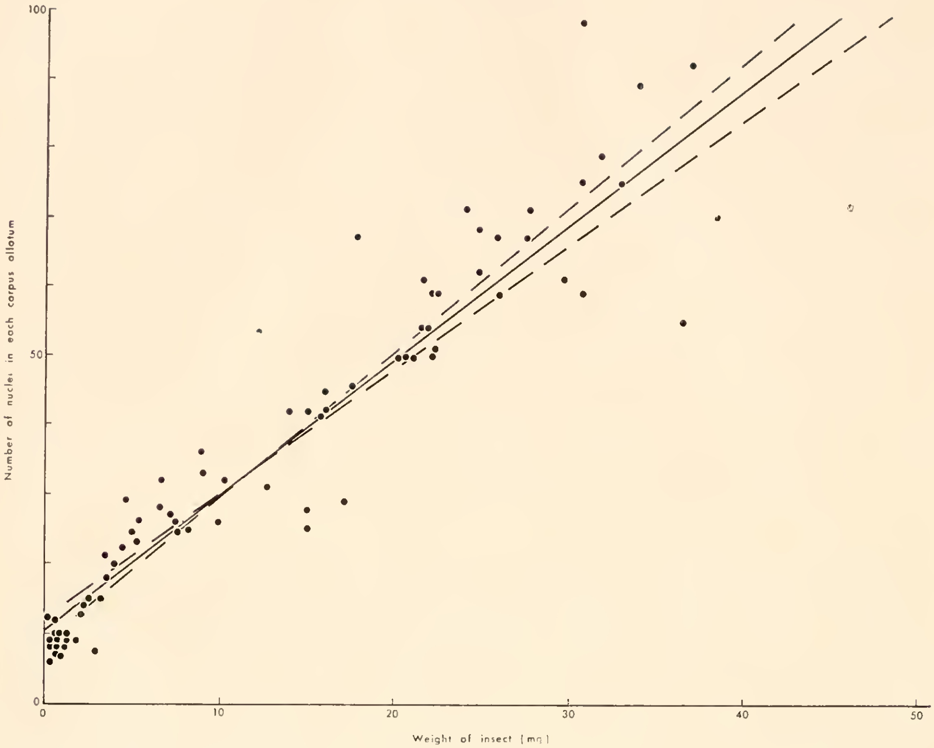

FIGURE 3. Relationship between the weight of a firebrat and the number of allatal cells. The fitted regression has the form: $y = 10.61 + 1.94x$ (modified after Watson, 1963a).

3 has the form:

$$y = 10.61 + 1.94x,$$

where y = the number of cells in each corpus allatum and x = the weight of the firebrat in mg. The 5% confidence limits for the regression coefficient are 1.80 and 2.08, so that the regression line does not intersect the origin, $P < 0.001$.

TABLE I

The numbers of allatal nuclei in larval Thermobia

Instar	Average	Range	N
1st	13.9	9.6–18.4	6
Early—mid-2nd	17.2	11.2–24.0	8
Late 2nd	16.9	15.1–18.6	5
Early 3rd	15.6	12.0–21.6	10
Mid-3rd	17.4	12.4–22.1	10
Late 3rd	18.5	14.0–23.5	5
Early 4th	16.2	12.4–18.6	6
Mid-4th	16.1	12.4–19.7	11

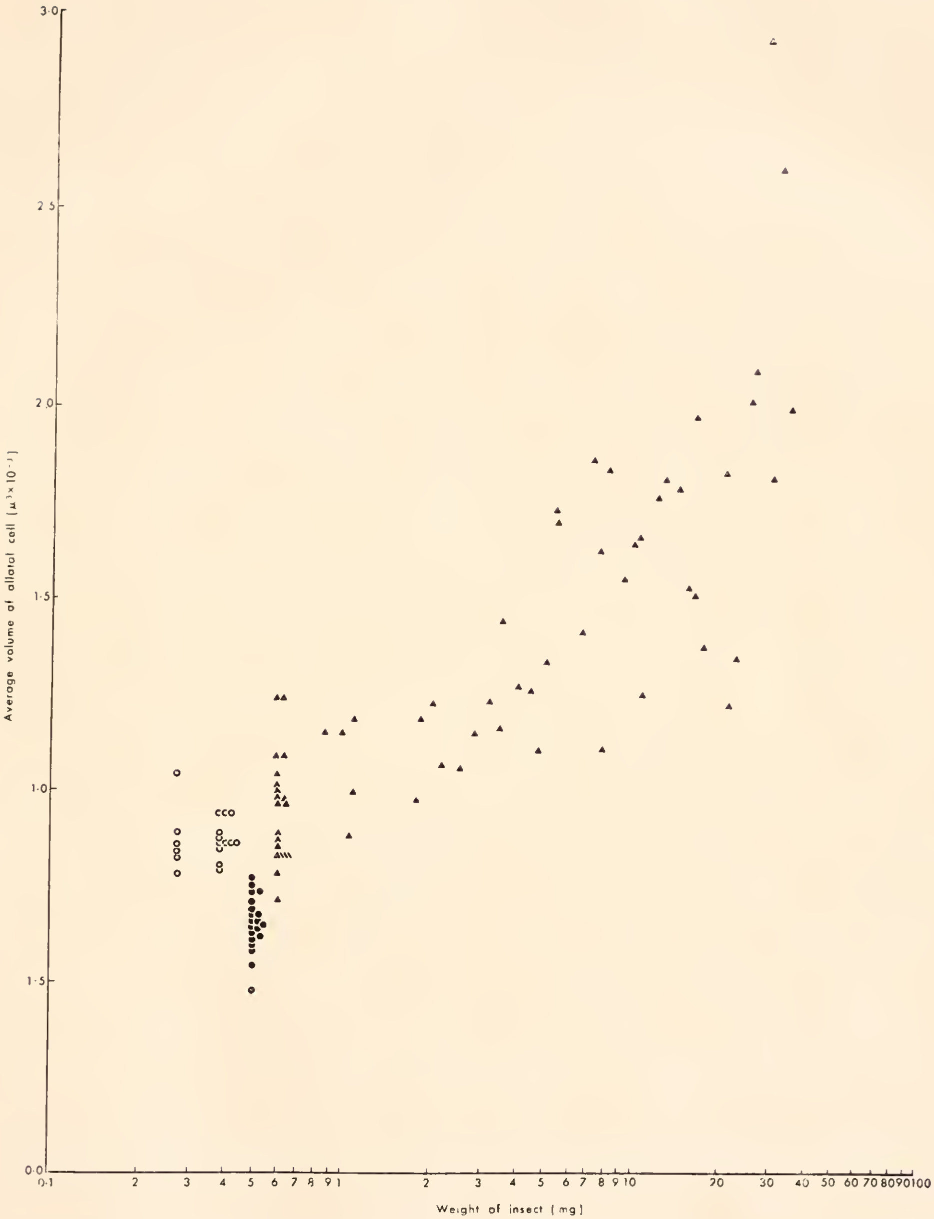

FIGURE 4. Changes in volume of allatal cells during the life of *Thermobia*. (Volumes for each of the first four instars are grouped sequentially, rather than by actual weight.) ○ = 1st and 2nd instar; ● = 3rd instar; ▲ = 4th and later instars.

Not only is the net growth in the corpus allatum linearly related to the size of the firebrat, but the growth is progressive; there are no cycles of increase and decrease within a stadium. Watson (unpublished results) has documented this for the adult, and an analysis for the first four larval stages appears in Table 1. None of the samples differs significantly from any other within that instar (Mann-Whitney "U" test).

(b) The volume of the corpus allatum

In addition to the changes in allatal volume due to the progressive increase in the number of allatal cells, there are changes due to fluctuations in the volumes of existing cells. As mentioned above, these changes do not involve nuclear volume, which remains constant throughout, but depend on increases and decreases in the amount of cytoplasm. The relevant data appear in Figures 4 and 5; Figure 4 shows the period from hatching through adult life (including reproducing females) and Figure 5 presents the early larval life in detail.

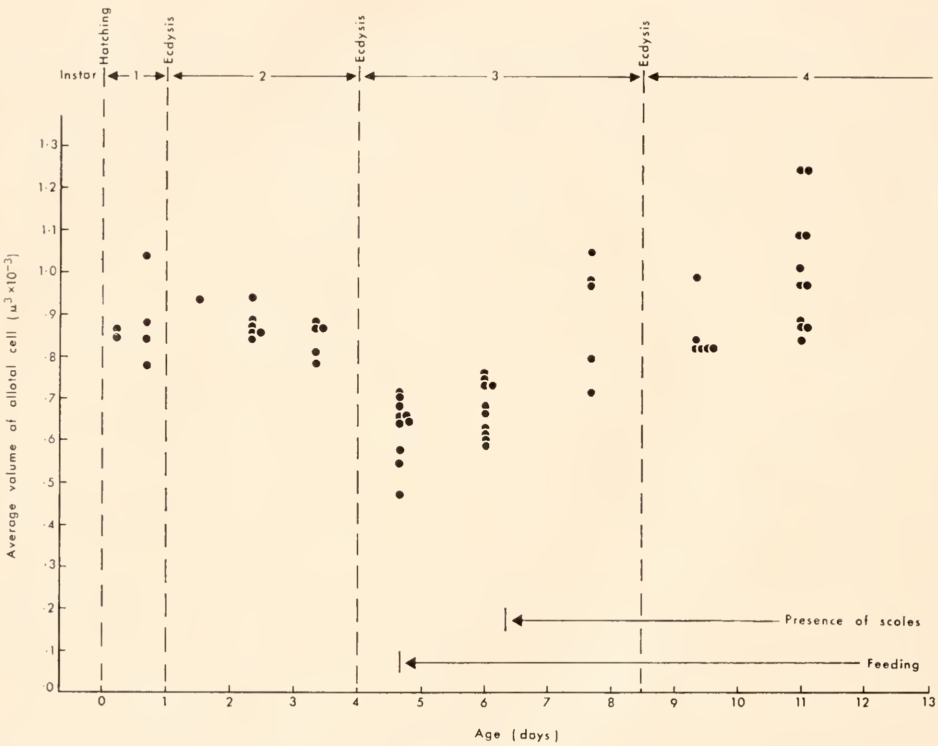

FIGURE 5. Changes in volume of allatal cells during the first four stadia of the firebrat.

The data document two points. First of all, once the scales have formed, the volume of cytoplasm in each allatal cell increases steadily until the attainment of sexual maturity. Thus the cell volumes in each of the categories, 4th instar — < 5 mg., >5 mg. – < 10 mg., and > 10 mg. (excluding mated females) (Fig. 4) exceed those in the previous category, $P < 0.002$ (Mann-Whitney "U" test). At weights of 8–10 mg., when the females start to mate, the cell volume may increase further, correlated with the development of the eggs (Watson, 1965 and unpublished results).

Secondly, there are changes in the allatal cytoplasm during the first four stadia. During the first two larval stages, the corpora allata maintain a constant cell volume (Fig. 5; $P > 0.10$, Mann-Whitney "U" test). After the second ecdysis, however, the volume of cytoplasm decreases; the cell volumes for early and mid-third stage larvae differ from those for first and second stage larvae, $P < 0.001$ (Mann-Whitney "U" test), as do the ratios between cytoplasm and nucleus. After the mid-third stage, when the scales have appeared on the new cuticle, the volume increases again; the cell volumes and ratios of cytoplasm to nucleus for the late third stage exceed those for the mid-third stage, $P < 0.01 > 0.001$, but do not differ from those of the fourth stage, $P > 0.10$ (Mann-Whitney "U" test).

The question then arises: Do these changes in the volume of the corpus allatum reflect changes in the physiological activity of the gland?

4. *The juvenile hormone activity of the corpora allata*

An initial series of assays based on the implantation of either maxillae or the ventral halves of the head into the left antenna of Polyphemus pupae with body or dorsal cephalic tissue as control in the right antenna, showed that the corpora allata from first stage and fourth or fifth stage larvae and from young adults possess juvenile hormone activity (Table II). Measured over the entire sample, the allatal activity exceeded that shown by an equal or greater amount of body tissue ($P = 0.0154$, Fisher exact probability test). The antennae of the resulting adult moths generally showed slight to considerable thickening of the midrib in the vicinity of the allatal implant, with fusion of the barbs up to half the width of the antenna, equivalent to scores of 0 to 3 on the scales of Gilbert and Schneiderman (1960). In the right antenna, only bodies from the first stage showed any juvenile hormone activity. In no case was there any general, systematic effect such as Yashika (1960) obtained with *Ctenolepisma*, but Yashika implanted far greater quantities of allatal tissue, and into the abdominal hemocoel.

TABLE II

The distribution of juvenile hormone activity in heads and body fragments of Thermobia

Stage of donor	Implant		No. adults emerged	L + ve	R + ve	L > R
	L. antenna	R. antenna				
1st	Ventral head	Body fragments	11	6	2	5
4th–5th	Ventral head	Body fragments	13	7	0	7
Adults	Maxillae	Dorsal head or labium	4	3	0	3

The experiment was then extended to assay the activity of early larval corpora allata. The ventral halves of five heads were implanted into each antenna, a different instar being used as donor for each side. The antennae of the resulting adult were scored as above, and the differences between the two antennae were analyzed by a sign test; the magnitude of the differences was disregarded, as the scoring table is based on an ordinal scale.

The results appear on Table III. As the Table shows, the corpora allata reach their minimal activity early in the third stage; the activities of the first and second stages do not differ from each other; and there is a marked rise in activity between the third and fourth stages. Thus the juvenilizing activity of the corpus allatum is related to the volume of cytoplasm in the gland. In other words, the activity of the corpus allatum is minimal at the time that scale formation is induced.

5. *The regulation of the corpus allatum*

The coincidence between the exhaustion of yolk reserves and the minimal activity of the corpus allatum suggests that the two may be connected; the activity of the corpus allatum may be controlled, directly or indirectly, by the quantity or quality of food.

TABLE III

Juvenile hormone activity in the corpora allata of early larval Thermobia

Comparison		L < R	L = R	L > R	Interpretation and probability (Sign test)
L. antenna	R. antenna				
1st	2nd	6	4	1	2nd $\geqslant$ 1st: $P = 0.062$
2nd	early 3rd	1	3	7	2nd > early 3rd: $P = 0.035$
early 3rd	late 3rd	8	1	2	Late 3rd $\geqslant$ early 3rd: $P = 0.055$
early 3rd	4th	11	1	0	4th > early 3rd: $P = 0.006$

The allatal volume was therefore measured in third stage larval firebrats that were isolated immediately after ecdysis, placed under optimal physical conditions and provided only with cellulose ("starved" firebrats). It was found that such larvae generally became moribund and died within four days, despite the fact that cellulose was ingested; none survived to molt into the fourth stage. The corpora allata were much smaller in the "starved" firebrats than in normal animals of the same age, attaining a constant minimal volume within 48–60 hours after ecdysis (Fig. 6); $P = 0.004$ (Mann-Whitney "U" test).

Third instar firebrats that had been fed on cellulose for 3 or 4 days were then fed with cereal containing protein, and the allatal volumes were measured 24, 48 and 72 hours after resumption of normal diet, and immediately after ecdysis to the fourth stage which occurs about 10 days after the second ecdysis. As the data in Figure 6 show, an increase in allatal volume followed the resumption of feeding, except in animals which had become too moribund to recover (*e.g.* low values at 48 and 72 hours). The overall reduction in allatal volume persisted at least until ecdysis to the fourth stage, at which time the corpora allata were still smaller than in control animals (Fig. 5), $P = 0.012$ (Mann-Whitney "U" test), although they exceeded the volumes at the end of starvation, $P = 0.018$ (Mann-Whitney "U" test).

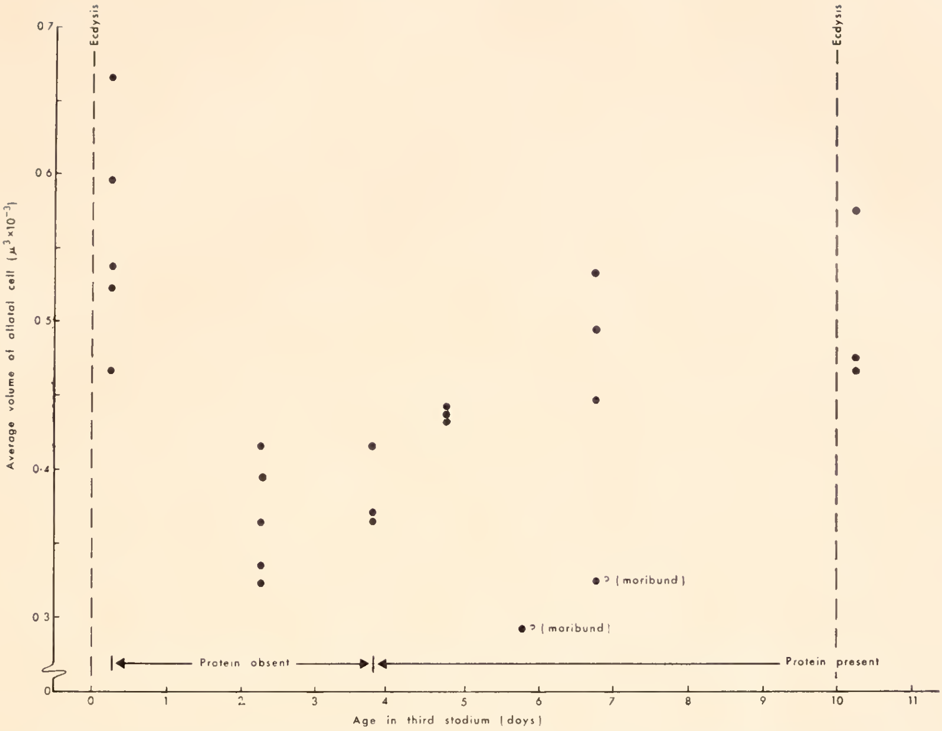

FIGURE 6. The effect of starvation on the allatal volume of 3rd instar *Thermobia*.

Thus irrespective of whether the effect of starvation is greater on the corpus allatum than it is on other tissues, it is evident that nutrition can influence the volume of allatal cytoplasm and hence, the physiological activity of the gland.

DISCUSSION

The data presented above strengthen the suggestion of Piepho and Richter (1959) and Richter (1962) that the post-embryonic development of lepismatids is regulated by changing titers of juvenile hormone. More specifically, one may correlate the appearance of scales during the third molting cycle with a precipitate fall in the activity of the corpora allata at the time of the second ecdysis, a fall possibly connected with the exhaustion of food reserves.

The question then arises: Why should scales persist when the allatal activity increases in later instars?

It is impossible to make any precise estimates of what the actual titers of juvenile hormone might be. If, however, it is assumed that the secretory potential of the corpus allatum is directly related to the volume of its cytoplasm, a supposition which is probably an oversimplification, but about which there is little critical information, it follows that the maximal titer of juvenile hormone that the gland can produce will depend on the volume of cytoplasm per unit volume of insect (*cf.*

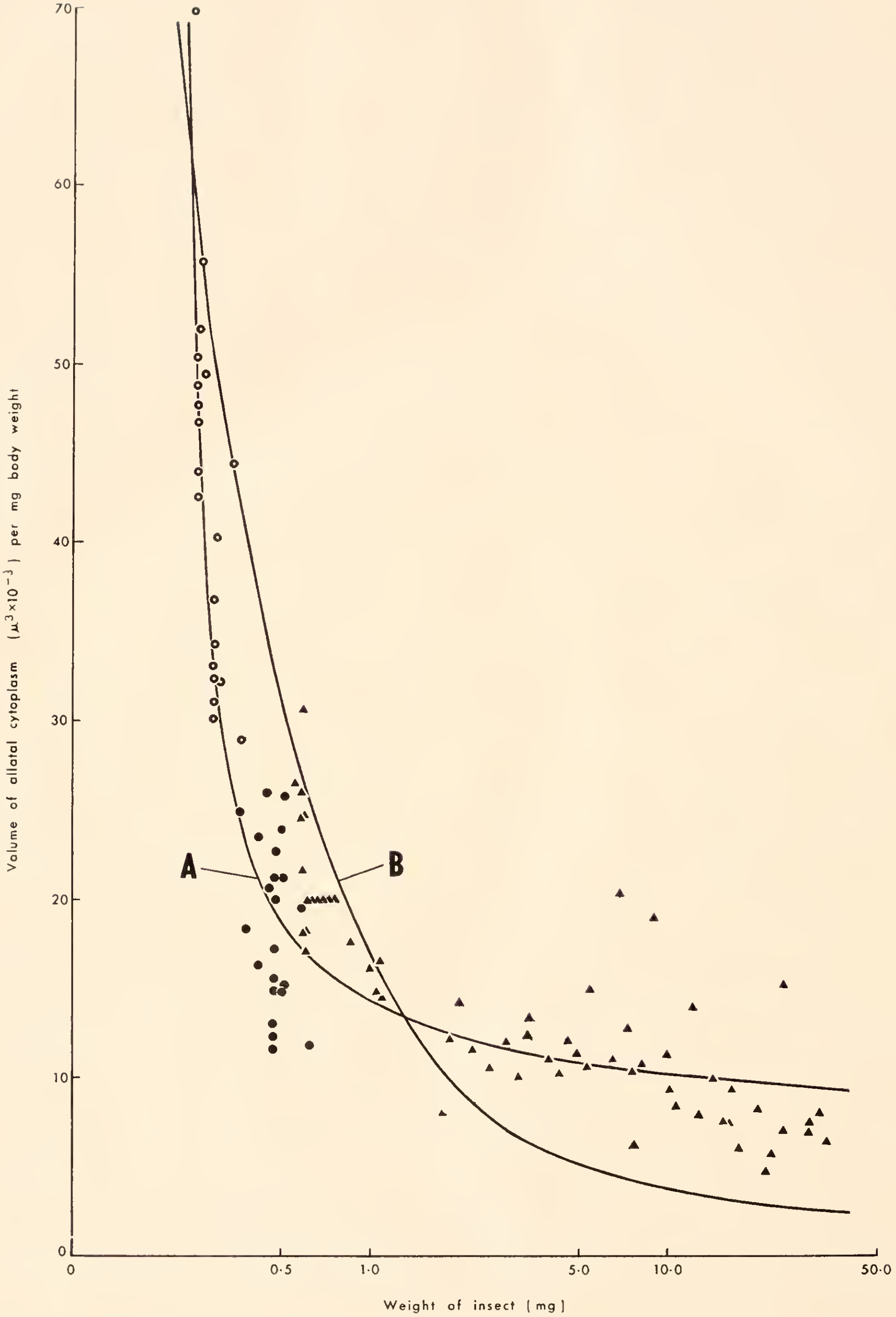

FIGURE 7. Changes in the amount of allatal cytoplasm per mg. body weight during the life of *Thermobia*. For the interpretation of curves A and B see text. Symbols as in Figure 4.

Novak, 1954). Thus in *Thermobia* the maximal titer would depend on the number of allatal cells and the average volume of cytoplasm contained in each cell. The pattern of growth in the corpus allatum ensures that the number of cells per mg. body weight decreases rapidly as the larva increases in size. At hatching, there are approximately 63 allatal cells per mg. body weight, the number of allatal cells then averaging 13.9 (Table 1) and the weight, 0.22 mg.; in the mid-late third stage, the number has fallen to approximately 41 cells per mg., the weight then averaging 0.43 mg. and the number of allatal cells, 17.7; at sexual maturity, with a body weight of approximately 10 mg., there are some 6 allatal cells per mg.; and the number approaches the asymptotic value, 3.88, in old age, at weights of 50 mg. The decline in titer that this pattern of growth could provide is shown in Figure 7B, based on the median value for the volume of cytoplasm per allatal cell (1,358 μ^3) and the regression in section 3(a) above.

However, this decline is partially offset by the progressive increase in the volume of allatal cytoplasm. The observed values for the volume of allatal cytoplasm per mg. body weight appear in Figure 7 and curve A is fitted to these points. It is evident that despite the cytoplasmic changes, the range of values in the third instar is not exceeded in later larval or adult life, even in reproducing females.

Furthermore, even if the titer of juvenile hormone were to exceed the values of the early third stage, it seems probable from studies of higher insects that the dedifferentiation of the epidermal cells would not be immediate (*cf.* Wigglesworth, 1954; Lawrence, 1966). Thus inactivation of juvenile hormone from the onset of the molting cycle would prevent the prolonged exposure to the hormone which appears to be a prerequisite for dedifferentiation. It is therefore of interest that such inactivation occurs, at least in the adult firebrat; the activity of exogenous hormone (Cecropia extract) persists during the reproductive phase of the adult stadium, but not during the molting phase (Watson, unpublished results; *cf.* Watson, 1964a, 1964b).

I wish to acknowledge the hospitality of Professor H. A. Schneiderman, of the Department of Biology, Western Reserve University; and the financial assistance of the Lalor Foundation and the Queen Elizabeth II Fellowships Committee, together with grants from the National Institutes of Health and the National Science Foundation to Professor Schneiderman. I also wish to thank Mrs. M. Hudack for her help with the histology.

SUMMARY

1. The integument of the firebrat, *Thermobia domestica*, lacks scales until the molt from the third to the fourth larval stage, but retains them in all subsequent instars.

2. Implantation experiments confirm earlier findings that the scaleless integument of first stage larvae will develop scales prematurely when implanted into a molting adult, implying that the formation of scales is humorally determined.

3. The number of cells in the corpora allata increases progressively throughout the life of the firebrat, and the relationship between the number of allatal cells and the weight of the firebrat is expressed by the regression:

$$y = 10.61 + 1.94x,$$

where y = the number of cells in each corpus allatum and x = the weight of the insect in mg. The regression does not intersect the origin.

4. The size of nuclei in the corpus allatum remains constant throughout life, but the volume of cytoplasm can alter. The minimal cytoplasmic volume coincides with the deposition of the first scale-bearing cuticle. The amount of cytoplasm then increases abruptly, the level continuing to rise slowly throughout the rest of larval life. A further increase may occur in mated females.

5. The juvenile hormone activity of the corpora allata, when assayed on pupae of the silkmoth *Antheraea polyphemus,* correlates with the volume of cytoplasm in the gland, and is minimal in the third stage.

6. The activity of the corpora allata is influenced by the intake of food, specifically protein.

7. Dedifferentiation of the epidermis is prevented by the pattern of growth in the corpus allatum, which indicates that the secretory potential of the gland, expressed as the volume of allatal cytoplasm per milligram body weight, is greater during the first through third instars than at any later stage, and by the inactivation of juvenile hormone during the molting cycle.

LITERATURE CITED

Bailey, N. T. J., 1959. Statistical Methods in Biology. English Universities Press, London.

Delany, M. J., 1957. Life histories in the *Thysanura. Acta Zool. Cracov.,* **2**: 61–90.

Gilbert, L. I., and H. A. Schneiderman, 1960. The development of a bioassay for the juvenile hormone of insects. *Trans. Amer. Micr. Soc.,* **79**: 38–67.

Lawrence, P. A., 1966. The hormonal control of the development of hairs and bristles in the milkweed bug, *Oncopeltus fasciatus,* Dall. *J. Exp. Biol.,* **44**: 507–522.

Marrable, A. W., 1962. The counting of cells and nuclei in microtome sections. *Quart. J. Micr. Sci.,* **103**: 331–347.

Novák, V. J. A., 1954. Growth of the corpora allata during the postembryonal development in insects. *Mem. Soc. Zool. Tchecosl.,* **18**: 98–133.

Piepho, H., and A. Richter, 1959. Zur Entwicklungsphysiologie des Schuppenkleides bei Ur-Insekten, Untersuchungen am Silberfischen *Lepisma saccharina* L. *Biol. Zbl.,* **78**: 855–861.

Pflugfelder, O., 1958. Entwicklungsphysiologie der Insekten. Geest u. Portig, Leipzig.

Richter, A., 1962. Über die Entwicklung der Schuppenorganen und der Genitalanhänge in Abhängigkeit vom Hormonsystem bei *Lepisma saccharina* L. *Arch. f. Entw.,* **154**: 1–28.

Rohdendorf, E., 1966. Der Einfluss der Allatektomie auf adulte Weibchen von *Thermobia domestica* PACKARD (Lepismatidae, Thysanura). *Zool. Jahrb. Physiol.,* **8**: 685–693.

Scharrer, B., 1964. Histophysiological studies on the corpus allatum of *Leucophaea maderae* IV. Ultrastructure during normal activity cycle. *Z. Zellforsch.,* **62**: 125–148.

Schmidt, E. M., 1959. Untersuchungen zur Cytologie des Wachstums in der postembryonalen Entwicklung von *Lepisma saccharina* L. *Zool. Jahrb. Anat.,* **77**: 357–410.

Siegel, S., 1956. Nonparametric Statistics for the Behavioral Sciences. McGraw-Hill Book Co., Inc., New York, Toronto and London.

Sweetman, H. L., 1952. The number of instars among the Thysanura as influenced by the environment. *Trans. 9th Int. Congress of Entomol.,* **1**: 411–415.

Watson, J. A. L., 1963a. The cephalic endocrine system in the Thysanura. *J. Morph.,* **113**: 359–373.

Watson, J. A. L., 1936b. Control of postembryonic development in the firebrat, *Thermobia domestica* (Packard). *Amer. Zool.,* **3**: 533.

Watson, J. A. L., 1964a. Moulting and reproduction in the adult firebrat, *Thermobia domestica* (Packard) (Thysanura, Lepismatidae)—I. The moulting cycle and its control. *J. Ins. Physiol.,* **10**: 305–317.

WATSON, J. A. L., 1964b. Moulting and reproduction in the adult firebrat, *Thermobia domestica* (Packard) (Thysanura, Lepismatidae)—II. The reproductive cycles. *J. Ins. Physiol.*, **10**: 399–408.

WATSON, J. A. L., 1965. The endocrine system of the lepismatid Thysanura and its phylogenetic significance. *Proc. 12th Int. Congress of Entomol.*, p. 144.

WIGGLESWORTH, V. B., 1954. The Physiology of Insect Metamorphosis. Cambridge University Press, Cambridge.

YASHIKA, K., 1960. Studies on the neurosecretory system in Apterygota. II. Development of the corpus allatum in *Ctenolepisma* and its juvenile action on the Philosamia-pupa. *Mem. Coll. Sci. Kyoto, Ser. B.*, **27**: 83–88.

LIGHT-INDUCTION OF SHEDDING OF GAMETES IN CIONA INTESTINALIS AND MOLGULA MANHATTENSIS [1]

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Castle (1896) and Conklin (1905) observed that the release of gametes in three species of solitary ascidians (*Ciona intestinalis*, *Styela partita* and *Molgula manhattensis*) occurred at definite times during the daylight period. *Ciona intestinalis* and *Molgula manhattensis* spawn one to one and a half hours before sunrise and *Styela partita* spawns during the late afternoon. Grave (1921, 1937) found, in the colonial ascidian *Amaroucium constellatum*, that the greatest release of larvae occurred at and just before sunrise but they continued to be liberated in small numbers throughout the daylight period. He reported a similar phenomenon in *Botryllus schlosseri;* in this colonial form the larvae are released in increasingly greater numbers as the day advances, finally reaching a maximum at noon. Rose (1939) recorded that *Styela partita* could be induced to shed their eggs by subjecting them to 11–12 hours of light prior to the desired time of spawning. He observed that the natural time for shedding occurred in the laboratory between 4 and 7 PM. Furthermore, spawning under experimental conditions could be induced on four or five successive days by controlling the illumination. As yet there has been no study made of the characteristics of the illumination necessary to cause shedding.

In the present study the effects of the intensity and the wave-length of light upon shedding have been investigated in two solitary oviparous ascidians which shed at dawn—*Ciona intestinalis* and *Molgula manhattensis*. Before this was done, however, it was necessary to determine the exact times of shedding in these two species.

MATERIALS AND METHOD

Ciona intestinalis and *Molgula manhattensis* were obtained from the supply department at the Marine Biological Laboratory, Woods Hole, Massachusetts, during the months of June, July and August, 1965. These animals were kept for several days in laboratory aquaria; those ascidians which were sexually mature and shedding gametes were selected for experimental purposes, and after being subjected to a particular treatment they were discarded.

All experiments were conducted in a room where the ascidians were subjected to a standard day consisting of 12 hours of light and 12 hours of darkness. In order that two series of experiments could be carried out simultaneously, animals were kept in black-painted light-tight boxes during the period of 12 hours of darkness.

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[1] This work was carried out during the summer of 1965 at the Marine Biological Laboratory, Woods Hole, Massachusetts, while the author was a recipient of a Pennsylvania Plan Fellowship in the Department of Animal Biology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia. Support was made available through training grant 5T1-HD 26-04 from the National Institutes of Health.

In one experiment light was provided from 9 AM to 9 PM, while in the second experiment light was provided from 9 PM to 9 AM. In the method outlined above some of the experiments were carried out under conditions of lighting which were completely reversed from the conditions existing in the animals' natural habitat. No apparent differences were observed between animals shedding at approximately their normal time and those shedding in the reversed system of lighting.

The ascidians were kept individually in fingerbowls containing 250 ml. of filtered sea water which was changed every 12 hours. The temperature of the sea water was recorded at the time it was placed in the fingerbowl and at the end of the 12-hour period just before it was replenished. The fingerbowls were placed on a black surface to keep the reflection of light from the source to a minimum. (Reflecting power of a black painted matt surface is less than 1%.) The shedding response was recorded as either positive when gametes were released or negative when no gametes were shed.

Intensity

In the first series of light-intensity experiments, a standard light source—100-watt bulb—was used and the intensity varied by placing the ascidians at different distances from the light source. The intensity of illumination from a constant light source varies inversely as the square of the distance from that source. Theoretical values of light intensity at various distances from the light source may be calculated from the following equation:

$$\frac{\text{Intensity of illumination at A}}{\text{Intensity of illumination at B}} = \frac{d_B^2}{d_A^2}$$

where d_A and d_B are the distances of A and B from the light source, respectively, since manufacturers supply data on the intensity of illumination at 1 foot.

The shedding response was measured at 1 foot, 2 feet, 4 feet, and 8 feet from the light source and the intensity of illumination was measured with a light meter to check the theoretical calculations. Groups of six animals were placed in each treatment and their shedding responses recorded on four successive days. In these experiments the time when shedding occurred after the animals were introduced into the light was tabulated for both species of ascidians.

In a second series of light-intensity experiments four different light sources—60-watt, 40-watt, 25-watt, and 15-watt bulbs—were used at a standard distance of eight feet from the animals.

Wave-length

Four Kodak Wratten gelatin filters were used to determine the effects of the wave-length of light upon shedding. The four filters had the following characteristics: (1) Filter No. 2B absorbed light at wave-lengths of 390 mμ and below, the approximate colors absorbed were ultraviolet and violet light. (2) Filter No. 16 absorbed light at wave-lengths of 500 mμ and below, the approximate color range absorbed being blue, blue green, and those colors absorbed by filter No. 2B. (3) Filter No. 25 absorbed light at wave-lengths of 600 mμ and below, the approximate color range absorbed being green and yellow plus those colors absorbed by filter

No. 16. (4) Filter No. 89B absorbed light at wave-lengths of 700 mμ and below, the approximate color range absorbed being orange and red plus those colors absorbed by filter No. 25. Each filter measured 10 cm. by 12 cm. and it was placed in a darkroom safelight. A fixed intensity of 32 foot-candles was used; this intensity had been found to produce optimum shedding responses in previous studies on the effect of light-intensity on this process. Shedding responses were recorded on two successive days. Three separate trials were made with *Ciona intestinalis;* in each trial nine animals were allotted to a treatment. *Molgula manhattensis* was not as plentiful and 6 animals were used per treatment and 2 trials were made.

RESULTS AND DISCUSSION

1. *Time of shedding*

In the first series of light-intensity experiments the time when shedding commenced after the ascidians were introduced into light was recorded. The data for *Ciona intestinalis* and *Molgula manhattensis* are presented in Tables I and II, respectively. In each species 24 animals were allotted at random to the 4 light intensities so that the responses of 6 animals were observed at each light-intensity. The animals were stimulated to shed on 4 occasions, 24 hours apart. Mean shedding times at the 4 light-intensities are given for each group of 6 animals on the 4 days when shedding responses were observed. In addition, the number of animals in these groups giving positive shedding responses on each occasion is tabulated. In *Ciona intestinalis* analyses of variance showed no significant differences in the shedding time of the animals between 4 levels of light-intensity. A similar analysis of the data from *Molgula manhattensis* indicated a difference in the shedding time of the animals between intensities on the first day (0.01 > P > 0.001) but no differences were found on the three subsequent days. At the highest light-intensity (130 foot-candles) the mean time taken for the 6 animals to shed after exposure to light (16.2 min.) was less than the mean times for the three lower intensities (26.5 min., 23.2 min., and 22.2 min.). It seems unlikely that this is a true effect of light-

TABLE I

Mean shedding times in minutes for Ciona intestinalis recorded at 4 different intensities of light

Six animals per light-intensity, shedding responses recorded on 4 consecutive days

Day	1		2		3		4	
Light intensity (foot-candles)	Number shedding	Mean shedding time	Number shedding	Mean shedding time	Number shedding	Mean shedding time	Number shedding	Mean shedding time
130	6	3.67	6	3.17	4	2.5	4	3.75
32	6	7.17	5	5.2	5	3.6	5	6.8
8	4	3.0	4	2.25	4	3.5	5	6.0
15	2	2.0	3	4.0	5	2.6	3	2.67
Total number shedding	18		18		18		17	
Mean shedding time		4.5 ±3.22		3.67 ±1.87		3.06 ±1.11		5.12 ±3.33

Mean shedding time for 24 animals during 4-day period—4.07 min. ±2.60.

TABLE II

Mean shedding times, in minutes for Molgula manhattensis recorded at 4 different intensities of light

Six animals per light-intensity, shedding responses recorded on 4 consecutive days

Day	1		2		3		4	
Light intensity (foot-candles)	Number shedding	Mean shedding time	Number shedding	Mean shedding time	Number shedding	Mean shedding time	Number shedding	Mean shedding time
130	5	16.2	5	23.8	2	28.5	2	27.5
32	6	26.5	6	23.67	6	25.5	1	31.0
8	6	23.2	4	27.75	4	26.25	2	26.0
1.5	5	22.2	4	24.25	5	24.0	5	22.2
Total number shedding	22		19		17		10	
Mean shedding time		22.78 ±5.36		24.68 ±3.53		25.59 ±3.98		24.9 ±3.73

Mean shedding time for 24 animals during the 4-day period—24.16 min. ±4.46.

intensity upon the time of shedding since it is not repeated on the subsequent days; however, these animals may have adapted themselves to this high intensity of light on the latter three days of the experiment.

Mean shedding times for both species were calculated from the data obtained during the whole experiment. In *Ciona intestinalis* shedding occurred in 22/24 of the animals used and 74% (71/96) positive shedding responses were obtained

TABLE III

Shedding response patterns of Ciona intestinalis and Molgula manhattensis observed on 4 consecutive days

Shedding response patterns Day					*Ciona intestinalis* Light-intensities					*Molgula manhattensis* Light-intensities (Foot-candles)				
Type	1	2	3	4	130	32	8	1.5	Total	130	32	8	1.5	Total
1	1	1	1	1	4	5	3		12	1	1	1	3	6
2	1	1	1	0						1	5	2	1	9
3	1	1	0	1						1				1
4	1	1	0	0	2				2	1		1		2
5	1	0	1	1			1	1	2			1	1	2
6	1	0	1	0				1	1					
7	1	0	0	1										
8	1	0	0	0		1			1	1		1		2
9	0	1	1	1				2	2					
10	0	1	1	0				1	1					
11	0	1	0	1			1		1					
12	0	1	0	0						1				1
13	0	0	1	1										
14	0	0	1	0										
15	0	0	0	1										
16	0	0	0	0			1	1	2				1	1

1 = shedding
0 = no shedding

during the 4 days. This species commenced shedding gametes 4.07 min. ± 2.60 after exposure to light. In *Molgula manhattensis* 23/24 animals shed and 71% (68/96) positive shedding responses were obtained during the 4 days; the overall mean time when shedding commenced was 24.16 min. ± 4.46 after exposure to light. Therefore, from the data above it has been shown that these two species of ascidians have their own characteristic shedding time. If the light stimulus mediates its effect *via* the neural ganglion and neural gland complex of these animals, the response of these structures to the light-stimulus occurs much more rapidly in *Ciona intestinalis* than in *Molgula manhattensis*.

Since the behavior of each animal was observed on 4 successive days its overall response pattern can be represented by a vector of 4 elements, 1 and 0 representing shedding and not shedding, respectively. There are thus 16 possible response patterns. The distribution of the shedding response patterns of the two species observed on the four consecutive days is listed in Table III. Fifty per cent (12/24) of *Ciona intestinalis* shed on four days whereas only 25% of *Molgula manhattensis* shed consecutively over a similar period. The highest shedding responses occurred on the first two days of the experiment; the fall in response over the last two days may have been due to the lack of food materials or to the handling of the animals.

TABLE IV

Percentage of Ciona intestinalis and Molgula manhattensis shedding in response to different light-intensities

Light intensities (foot-candles)	Number of animals	% Shedding	
		Ciona intestinalis	*Molgula manhattensis*
130	6	100.00	83.33
32	6	83.33	83.33
8	6	66.67	66.67
1.5	6	50.00	66.67
1.17	12	41.67	58.33
0.67	12	41.67	66.67
0.40	12	16.67	58.33
0.21	12	0	25.00

2. *Light-intensity*

The shedding responses of the two ascidians to 8 different light-intensities are presented in Table IV. These responses were recorded on the second day of exposure to the various light-intensities. The high intensities of light did not inhibit the shedding response. In *Ciona intestinalis* shedding was reduced to 16.67% at 0.40 foot candle and was completely inhibited at 0.21 foot-candle. The response in *Molgula manhattensis* was reduced to 25% at the latter light-intensity. The active contractions of the animals associated with the light-stimulus (Hecht, 1926) and the shedding of gametes (Castle, 1896) were diminished in both species at the lowest light-intensity (0.21 foot-candle). It may be suggested that the release of gametes is a reflex associated with the muscular contractions of the animal stimulated by exposure to light.

In the present study, it has been shown that these species shed at low as well as high light-intensities which would allow them to adapt to a fairly wide range of naturally occurring habitats (Van Name, 1945).

3. *Wave-length*

The data obtained showing the shedding response of the two species when the wave-lengths of light were restricted to certain regions of the spectrum are presented in Table V. In both species the shedding response was reduced to below 50% when light was absorbed up to 600 mμ and totally inhibited when absorbed up to 700 mμ. These results indicate that the excitatory wave-lengths occur between 500 mμ and 700 mμ. A constant light-intensity was used in these experiments—32 foot-candles—and therefore no interaction between light-intensity and wave-length was shown; however, further investigation of this aspect may help in the understanding of the shedding phenomenon. In the experiments described, the wave-length range which stimulates shedding has good transmission through sea water (Jerlov, 1964) and these wave-lengths would reach the animals in their natural habitat.

TABLE V

Percentage of Ciona intestinalis and Molgula manhattensis shedding in response to restricted wave-lengths of light

Wave-length absorption	*Ciona intestinalis*		*Molgula manhattensis*	
	Number of animals	% Shedding	Number of animals	% Shedding
390 mμ and below	27	81.84	12	91.67
500 mμ and below	27	62.96	12	83.33
600 mμ and below	27	18.52	12	33.33
700 mμ and below	27	0	12	0

The author gratefully acknowledges the advice and guidance given to him by Dr. J. D. Biggers during the course of the work and in the preparation of the manuscript.

SUMMARY

1. The time of shedding of gametes and the effects of the intensity and the wave-length of light upon the process have been investigated in *Ciona intestinalis* and *Molgula manhattensis*.

2. The time of shedding was recorded from 24 animals in each species on four consecutive days. It was found that *Ciona intestinalis* and *Molgula manhattensis* commenced to shed their gametes 4.07 min. ± 2.60 and 24.16 min. ± 4.46, respectively, after exposure to light. Twelve out of 24 *Ciona intestinalis* shed on 4 consecutive days and 6 out of 24 *Molgula manhattensis* shed consecutively over a similar period.

3. Intensities ranging from 0.21 to 130 foot-candles (f.c.) were used to study light-intensity effects. High intensities did not inhibit shedding but lower intensities —0.67 f.c. and 0.40 f.c.—produced a marked reduction in the shedding response. At 0.21 f.c. shedding was completely inhibited in *Ciona intestinalis* and reduced to 25% in *Molgula manhattensis*.

4. In both species, shedding was partially inhibited when light was absorbed up to 600 mμ and totally inhibited when absorbed to 700 mμ. This indicated that the excitatory wave-lengths lie between 500 mμ to 700 mμ.

LITERATURE CITED

Castle, W. E., 1896. The early embryology of *Ciona intestinalis* (L). *Bull. Mus. Comp. Zool.*, **27**: 201–280.

Conklin, E. G., 1905. Organization and cell lineage of the ascidian egg. *J. Acad. Nat. Sci., Philadelphia*, **13**: 1–21.

Grave, C., 1921. *Amaroucium constellatum* (Verrill) II. The structure and organization of the tadpole larva. *J. Morph.*, **36**: 71–91.

Grave, C., 1937. *In*: Culture Methods for Invertebrate Animals. Ed. Galtsoff *et al.*, Comstock, Ithaca, pp. 556–564.

Hecht, S., 1926. The effect of exposure period and temperature on the photosensory process in *Ciona*. *J. Gen. Physiol.*, **8**: 291–301.

Jerlov, N. G., 1964. Colour filters to simulate the extinction of daylight in the sea. *Extrait du Journal du Conseil International Pour l'Exploration de la mer,* **20**: 156–159.

Rose, S. M., 1939. Embryonic induction in the ascidians. *Biol. Bull.*, **77**: 216–232.

Van Name, W. E., 1945. The North and South American ascidians. *Bull. Amer. Mus. Nat. Hist.*, **84**: 1–476.

Vol. 132, No. 3 June, 1967

THE

BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

AMYLASE AND GLYCOGENOLYSIS IN AMPHIBIAN DEVELOPMENT[1]

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Since the carbohydrate reserves in amphibian eggs are mainly stored as glycogen (Gregg, 1948), attempts have been made to estimate its utilization during development as a function of glycogen breakdown. The most reliable data show that glycogen level begins to decrease when time of gastrulation starts (Brachet and Needham, 1935; Gregg, 1948). This has been confirmed by microchemical (Heatley and Lindahl, 1937; Jaeger, 1945) as well as histochemical methods (Woerdemann, 1933; Raven, 1935) which have also shown that glycogenolysis is stronger in those cells involved in the morphogenetic movements of gastrulation. Besides, the determination of the respiratory quotient (R.Q.) has given values concordant with those results. It is true that Barth (1946) has found a constant value of about 0.9; but Brachet (1934) and Boell (1955) have reported low values during segmentation, with a tendency to increase up to a value close to 1 at the time of gastrulation.

Less is known about the egg enzymes involved in glycogenolysis. Some evidence was reported indicating that glycogen breakdown could be accomplished through phosphoroclastic (Cohen, 1954; Gregg *et al.*, 1964) as well as through amylolytic pathways (see Urbani, 1962, for a review of the subject). The most outstanding news reported by the Italian author was the description of a β-amylasic activity in eggs of *Rana esculenta* and *Bufo vulgaris*.

Nothing is known, however, about glycogenolytic enzymes in *Bufo arenarum*. The limited information we possess on glycogen utilization during development agrees with the above reported results. Thus, a glycogen loss in eggs could be detected after the onset of gastrulation (Barbieri and Gil, 1962); and the R.Q. values were found to increase from about 0.6 during segmentation, up to 1 at the time of gastrulation (Legname and Barbieri, 1962).

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[1] This work was supported in part by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas (R. Argentina). 1

[2] Fellow of the Consejo Nacional de Investigaciones Científicas y Técnicas (R. Argentina) under the direction of Dr. J. M. Olavarría (Instituto de Química Biológica).

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The present paper has two purposes. The first is to report some additional data about glycogen contained in the eggs of the toad. The second is to present our first observations about the properties and biological role of an amylase found in the same material.

Material and Methods

Biological material

Oocytes of *Bufo arenarum* obtained by injection of pituitary glands preserved according to Pisanó (1956) were artificially fertilized. Development was allowed to proceed in 10% amphibian Ringer's solution without bicarbonate at laboratory temperature. Prior to homogenization, the jelly coat was dissolved by ultraviolet irradiation or by a neutralized thioglycolic acid solution.

Cellular fractionation

The eggs were homogenized, unless otherwise stated, with two volumes of chilled 0.25 *M* sucrose solution containing 0.001 *M* ethylenediamine-tetraacetic acid (disodium salt). The homogenization was carried out in an ice-cooled Potter type glass homogenizer. In order to establish the localization of glycogen and amylase, cellular fractions were isolated by differential centrifugation in Sorval RC-2 or Christ Universal refrigerated centrifuges. The homogenate was centrifuged, 20 minutes each time, at 1500 *g*, yielding a pellet containing nuclei, yolk platelets and pigment granules (fraction I), and at 10,000 and 25,000 *g*, sedimenting the mitochondria (fractions II and III). The remaining supernatant will be referred to as fraction IV.

Chemical methods

As a mild procedure for the extraction of glycogen, the $HgCl_2$ method of Mordoh *et al.* (1966) was adopted. A comparison of $HgCl_2$-extracted glycogen with cold trichloracetic acid-extracted glycogen, prepared from the same batch of eggs, has shown that they do not differ from the standpoint of the properties considered in this paper. As a standard method for the estimation of glycogen the phenol-sulfuric acid method as described by Dubois *et al.* (1956) was employed. The iodine reaction was performed in the presence of calcium chloride, according to Krisman (1962). Liver glycogen from adult specimens of the same species was used as a standard for both methods, and, in some experiments, it was extracted from the same females wherefrom the eggs used for analysis had been taken.

For the determination of amylase activity the reaction mixture contained, except where otherwise stated, 1.2 mg. of glycogen, 0.2 *M* phosphate buffer at pH 7.2, 0.1 *M* NaCl and 0.02 ml. of crude enzyme preparation, in a total volume of 0.08 ml. Incubation time was 20 minutes at 37° C., and the reaction was stopped by heating 2 minutes at 100° C. Reducing power was determined according to Somogyi and Nelson (Ashwell, 1957).

Absorption spectra of the color reactions with iodine were determined with a Beckman DU spectrophotometer, and the photometric readings with the phenol-sulfuric and Somogyi-Nelson methods were performed with a Spectronic 20 (Bausch & Lomb).

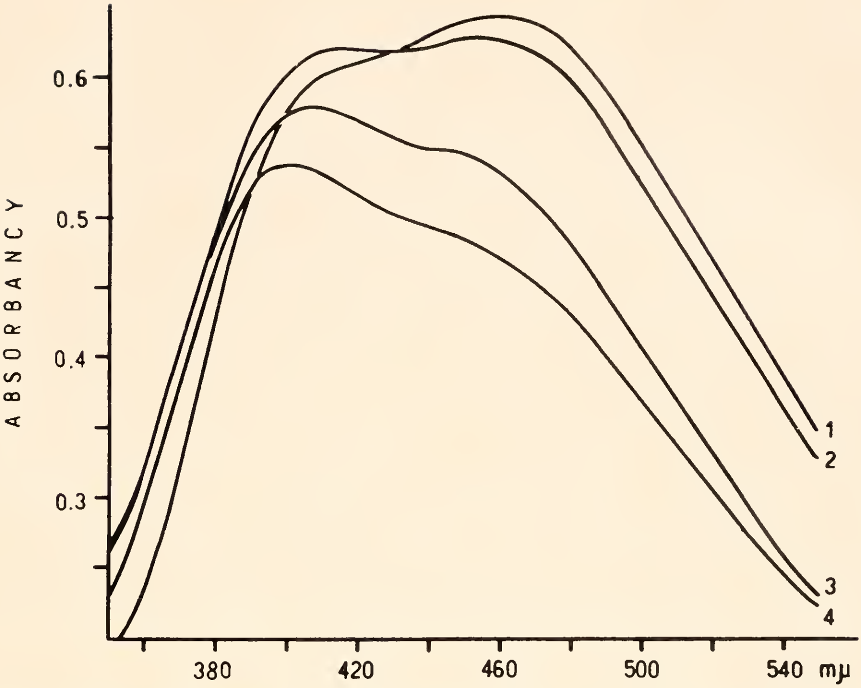


FIGURE 1. Absorption spectra of four glycogen preparations (0.05%) in the presence of iodine reagent. 1: unfertilized eggs; 2: rat liver; 3: toad liver; 4: tail bud embryos.

RESULTS

Glycogen

Egg glycogen dissolved in water never exhibits the milky appearance of the same concentration of liver particulate glycogen. Differential centrifugation of egg glycogen in aqueous solution, as well as its precipitation with varying concentrations of ethanol, allowed a rough estimate of its extensive polydispersity.

When studying the glycogen-iodine complex, egg glycogen was compared with liver glycogen of the same species as well as with rat liver glycogen prepared by

TABLE I

Changes in glycogen as shown by the iodine color reaction

Clutch	Stage	Iodine phenol-sulfuric	E_{400}/E_{460}
1	Second cleavage	0.98	1.09
	Neural fold	0.73	1.21
2	Unfertilized egg	1.02	0.98
	Tail bud	0.70	1.06
3	Unfertilized egg	1.09	0.97
	Tail bud	0.85	1.17

the same procedure. We found that the absorption spectrum of rat liver glycogen in the presence of iodine reagent presented, in addition to the absorption maximum at about 460 mμ as reported by Krisman (1962), a second λ_{max} at 410 mμ. Both maxima have about the same height and are separated by a slight depression (Fig. 1).

With respect to the toad, a significant difference could be detected between liver and oocyte glycogen. While liver glycogen showed a λ_{max} at about 400–410 mμ, oocyte glycogen presented a λ_{max} between 450 and 460 mμ (Fig. 1).

The affinity of glycogen for iodine was expressed by the ratio of the amounts of glycogen as determined by the iodine and the phenol-sulfuric acid methods, the value of this ratio being taken as 1 for the standard (Krisman, 1962). In the case of the iodine method, estimations were based on the average of extinctions at 400 and 460 mμ. The values of this ratio varied among different batches, but were generally slightly higher for oocytes than for liver glycogen.

When glycogen is extracted from developing eggs after gastrulation the absorption maximum shifts from 460 towards 400 mμ (Fig. 1). This displacement is expressed in Table I by the ratio of absorbancies at 400 and 460 mμ (E_{400}/E_{460}). Besides, the values of the ratio iodine/phenol-sulfuric indicate a fall of glycogen affinity for iodine over 20%. This change of affinity could also be detected in four glycogen fractions arbitrarily isolated by fractionated precipitation with ethanol (Fig. 2).

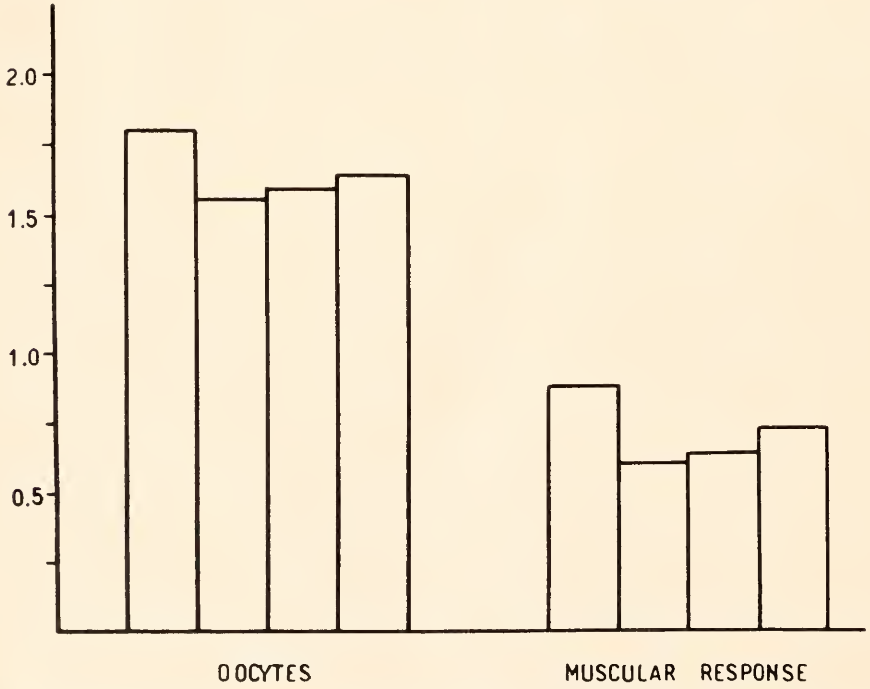
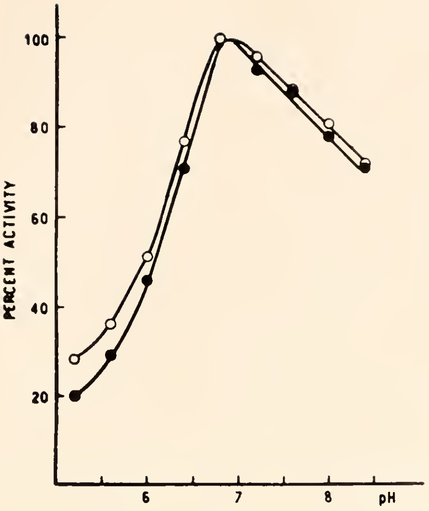


FIGURE 2. Histograms expressing the values of the iodine/phenol-sulfuric ratio in four glycogen fractions precipitated with 20, 30, 40 and 67% ethanol (reading from left to right).



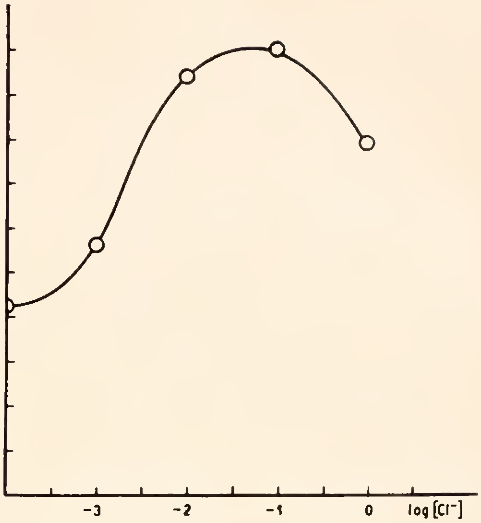


FIGURE 3 (left). pH optimum. Open circles: egg amylase; solid circles: mammalian salivary amylase.

FIGURE 4 (right). Effect of chloride on enzyme activity.

In connection with a point to be discussed later, it is important to add that egg glycogen does not seem to be associated to particles sedimenting as fraction I. In our working conditions, less than 10% of the total amount of glycogen could be detected in that fraction.

Amylase activity

The homogenates of *B. arenarum* eggs degrade glycogen in the same conditions as α-amylase. Thus, the pH of maximum activity is about 6.8 and it decreases sharply out of the range 6.4–7.8 (Fig. 3). The enzymatic activity is enhanced by chloride ions with an optimum concentration between 0.01 and 0.1 *M* (Fig. 4). Zn^{2+} is inhibitory and 10^{-5} *M* $HgCl_2$ showed no effect on the enzymatic activity (Table II). A Km value of 3.7 mg./ml. was found.

TABLE II

Effects of $ZnCl_2$ and $HgCl_2$ on amylase activity

Additions	Final concentration (*M*)	Enzyme activity*	
		Fraction I	Salivary amylase
$ZnCl_2$	10^{-3}	87	79
	10^{-2}	70	74
	10^{-1}	0	8
$HgCl_2$	10^{-5}	98	—

* Activity without salts taken as 100.

TABLE III

Recovery of amylase activity as a function of the procedure followed in the preparation of fraction I

Preparation	Enzyme activity (%)	
	Sediment	Supernatant
In 0.25 *M* sucrose	79	21
In 0.25 *M* sucrose washing once	2	98
In distilled water	29	71

After fractionation of oocyte homogenates, most of the activity appears localized in fraction I (nuclei, yolk and pigment granules), though in a very labile fashion. In fact, it suffices to wash the pellet only once with sucrose solution, or to homogenize the eggs with water, in order to loose the enzymatic activity of fraction I (Table III). We cannot decide as yet to which particles the enzyme is associated, but it does not seem to be linked to yolk platelets of major or medium size,

TABLE IV

Recovery of amylase activity from 0.25 M sucrose breis as a function of centrifugal force

Centrifugation	Enzyme activity (%)	
	Sediment	Supernatant
1500 *g*/20 min.	84	16
500 *g*/30 sec.	2	98

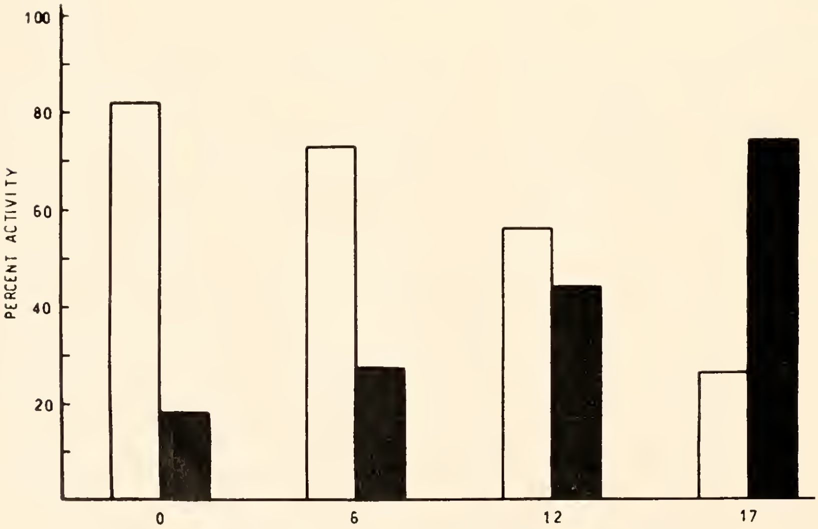


FIGURE 5. Enzyme activity of fractions I (nuclei-yolk-pigment) and IV (supernatant) as a function of developmental age. Abscissa, embryonic stages: 0, unfertilized eggs; 6, early cleavage; 12, gastrulation; 17, tail bud.

TABLE V

Activity of fraction I on glycogen as shown in vitro

Minutes of incubation	Reducing power (%)	Iodine/phenol-sulfuric	E_{400}/E_{460}
0	3	1.15	0.91
5	25	1.08	0.97
12	59	0.97	1.05
20	100	0.83	1.13

The incubation mixture contained: 2.4 mg. of glycogen and 0.05 ml. of enzyme in 0.004 *M* maleic acid-KOH buffer and 0.1 *M* NaCl (total volume 0.6 ml.).

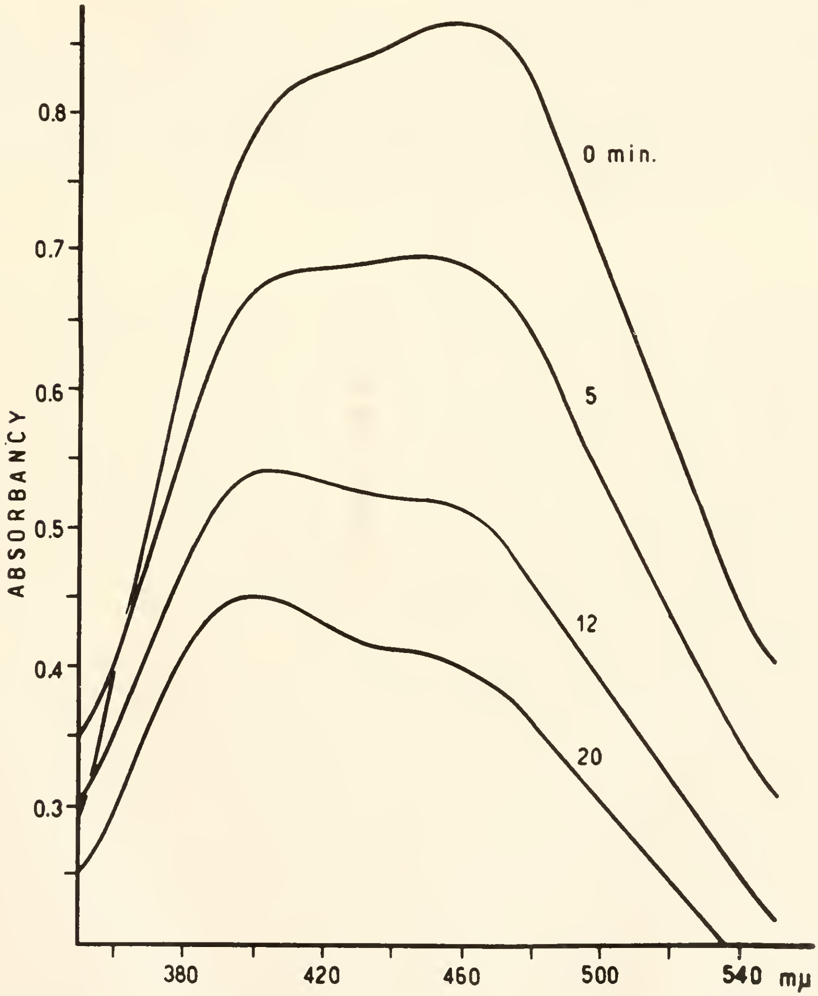


FIGURE 6. Glycogen breakdown by fraction I of unfertilized eggs, as shown by the iodine method. Incubation times are indicated in minutes.

since after sedimentation of most of the yolk mass at a low centrifugal field (500 *g* for 30 seconds) the activity remains in the supernatant (Table IV). Neither does it seem to be associated with the pigment granules, since an important part of these particles also sediments at 500 *g*.

Although the level of amylase activity remains constant up to the end of the neurula stages, the intracellular localization of the enzyme seems to change as a function of embryonic development (Fig. 5). While the activity of fraction I, which is the most important in the oocytes, decreases during development, the activity of fraction IV, *i.e.,* of the supernatant, increases at the same time. It is after gastrulation when the major part of the enzymatic activity appears in fraction IV at the expense of fraction I. The properties of this "soluble amylase" were found to be the same as those described for the enzyme linked to fraction I of the unfertilized egg.

The *in vitro* attack of glycogen by the enzyme of fraction I was also studied with the iodine color reaction. After incubating oocyte glycogen with a suspension of fraction I of the same origin, it was isolated by the $HgCl_2$ method and analyzed with the iodine reagent. The data collected in Table V and Figure 6 show that the optical properties of the glycogen iodine complex change in the same way after glycogen breakdown *in vivo* or *in vitro*.

Discussion

The iodine color reaction has proved useful to get some additional information about glycogen utilization in amphibian eggs. Let us point out first that the different absorption spectra reported for the toad and rat liver glycogens agree with previous observations indicating that the properties of the glycogen-iodine complex depend on the origin of the polysaccharide (Schlamowitz, 1951; Manners, 1957). Much more knowledge will be needed before the real basis of these differences may be understood; some evidence, however, is available showing that the iodine reaction reflects some structural aspects of the glycogen molecule. Thus, it has been shown that the value of the specific absorptivity coefficient is a function of the chain length and the value of the λ_{max} is related to the degree of branching of the polysaccharide (Swanson, 1948; Thoma and French, 1960; Archibald *et al.,* 1961; Bailey and Whelan, 1961). This holds also true for the reaction performed following the technique used in this paper (Krisman, 1962).

We have pointed out that after gastrulation, when a consumption of glycogen can be already detected, a fall of its affinity for iodine as well as a shift of the λ_{max} from 460 towards 400 mμ takes place. On the basis of Krisman's (1962) results, it can be assumed that a shortening of external branches has occurred. It is pertinent to observe that at this stage, when the iodine spectra of egg and liver glycogens become similar, we are dealing in both cases with actively metabolized glycogens. Oocyte glycogen, on the other hand, with a λ_{max} at 460 mμ, should be characterized by a relatively slow turnover rate. This last assumption finds some additional support in the following facts: (1) The apparent stability of glycogen level, even during egg segmentation (Barbieri and Gil, 1962); (2) the low respiratory activity with a R.Q. about 0.6 (Legname and Barbieri, 1962); (3) the negligible amounts of lactic acid contained in normal eggs (Barbieri and

Salomón, 1963). The apparent lack of activity exhibited by this cell and in this conection the widespread idea of viewing the unfertilized egg as an "anesthetized cell" (Brachet, 1960) should also be taken into account. Therefore, the iodine method seems to reveal, in the unfertilized egg of *B. arenarum,* the presence of a more "complete" or nearly "untouched" glycogen molecule.

We have found that glycogen isolated from eggs, as well as from several other sources, exhibits a high degree of polydispersity (Staudinger, 1948; Stetten *et al.,* 1956; Manners, 1957; Barber *et al.,* 1965; Mordoh *et al.,* 1966). The fact that most of its molecules, independently of their size, seem to be simultaneously metabolized, as shown by the iodine method, also agrees with previously reported results (Stetten and Stetten, 1960; Barber *et al.,* 1965).

Concerning our first observations in connection with the enzymes involved in the breakdown of egg glycogen we have established the presence of an enzyme with the properties of a mammalian α-amylase. Although only crude preparations have been used, some chromatographic controls of the reaction products, as well as the requirement of chloride ions, seem to exclude the presence of a relatively important glucosidase or phosphorylase activity in our working conditions.

It has already been mentioned that a β-amylasic activity has been described in eggs of *Rana esculenta* and *Bufo vulgaris* (Urbani, 1962), although no conclusive evidence has been provided. It is not unlikely that what has been taken for a β-amylase was really an α-amylase. During short incubation periods, such as those utilized by the Italian authors, α-amylase attacks only the outer branches of glycogen, with the formation of linear oligosaccharides (Olavarría and Torres, 1962). This means that during this first step of enzyme action, the analytical methods employed would not allow a clear-cut distinction between the two amylolytic pathways. Besides, while α-amylases exhibit a pH optimum between 6 and 7 and β-amylases an optimum below pH 6 (Fischer and Stein, 1960; French, 1960), "both" amylases of *B. vulgaris* eggs were found to have the same optimum at pH 7.2 (Scollo Lavizzari, 1956). We have also shown that the enzymatic activity of *B. arenarum* eggs remains unaffected in the presence of 10^{-5} M $HgCl_2$, which is known to inhibit β-amylase activity at that concentration. Finally, for the time being and at the present stage of our knowledge, β-amylases should be circumscribed to the plant kingdom (French, 1960).

A point of special interest concerns the intracellular localization of this enzyme. Løvtrup (1955), in *Amblystoma mexicanum* eggs, as well as Urbani and collaborators, in eggs of Anura, have found that the amylolytic activity should not be linked to yolk. Contrarily, as it has been shown, in the eggs of *B. arenarum* it should be associated to particles sedimenting at the same rate as the yolk platelets of minor size. This disagreement could be explained by the extreme lability of the enzyme-particle association. In fact, we have shown that if *B. arenarum* eggs are homogenized in water, as by the above-mentioned authors, most of the enzymatic activity remains in the supernatant after sedimentation of nuclei, yolk and pigment. In this connection, it is interesting to point out that when Urbani and Scollo Lavizzari (1955) measured amylase activity on parts of dissected embryos in the tail-bud stage they found the greatest activity in the portion richest in yolk; but as they found no activity in isolated yolk platelets, their conclusion was that the enzyme was localized in the protoplasm of the vitelline cells.

The observation that amylase seems to be progressively released from its supporting particle in the course of development has a promissory value concerning the intracellular localization of its substrate. Thus, on the assumption that glycogen is not linked to particles of fraction I, it is tempting to speculate that we are dealing with a regulatory mechanism of enzyme activity based on the spatial orientation of enzyme and substrate. It is true that glycogen could also be linked, in a very labile fashion, to the same particle to which amylase appears associated, but we do not count as yet with any experimental evidence supporting this possibility. Besides, there are reasons to believe that this regulatory mechanism is operative *in vivo:* (1) At the beginning of development, amylase activity appears restricted to fraction I; (2) as the activity of this fraction decreases throughout development, the activity of the supernatant increases (total activity remaining the same); (3) only one enzyme is involved in both fractions as far as we can judge, considering the properties analyzed in the present work; and (4) the changes suffered by glycogen, as shown by the iodine method, were the same after being attacked *in vivo* and *in vitro.*

Løvtrup (1955) has found that amylase activity in the non-yolk fraction of eggs of *Amblystoma* seems to increase through the stage of gastrulation, remaining unchanged once neurulation sets in. If we suppose that in these eggs the enzyme is linked to some particulate elements and that homogenization leads to a partial detachment of the former, we may assume that Løvtrup's graph representing "amylase synthesis" actually is an expression of the passage of the enzyme from the particles to the supernatant.

A regulatory enzymatic mechanism of this kind, depending upon the spatial orientation of enzyme and substrate, has already been proposed to explain the control of respiration in amphibian eggs (Spiegelman and Steinbach, 1945). It is not unlikely that such a mechanism was more generalized in these eggs than is currently believed. In this sense, our results give a new support to the view that yolk, more than as a simple reservoir of materials for the building up of the embryo, might function as an active part in metabolic control (Barth and Barth, 1954; Wallace, 1961).

Taking into account our limited knowledge about the function of amylase in adult tissues, as well as the important role that it seems to play in the breakdown of amphibian glycogen, further investigations along this line are being programmed.

Appreciation is expressed to Dr. Nicolás Bazán for his assistance at the beginning of this work, and to Dr. Peter Seeligmann for critical reading of the manuscript.

SUMMARY

1. An enzyme with the properties of a mammalian α-amylase in the eggs of the toad *Bufo arenarum* is described. The enzyme appears associated in a very labile fashion to particles sedimenting at 1500 g for 20 minutes in 0.25 M sucrose solution.

2. Egg glycogen is polydisperse and does not seem to be linked to the same particles to which amylase appears associated. At the beginning of development it reacts with iodine in a different way than liver glycogen of the same species, as

was shown by their absorption spectra; after gastrulation, when glycogen is supposed to be actively metabolized, the spectra of embryo and adult glycogens become similar.

3. Some evidence is presented indicating that egg glycogen in the course of development is degraded by the action of amylase, which would be progressively released from its compartment.

LITERATURE CITED

Archibald, A. R., I. D. Fleming, A. M. Liddle, D. J. Manners, G. A. Mercer and A. Wright, 1961. α-1,4-Glucosans. Part XI. The absorption spectra of glycogen- and amylopectin-iodine complexes. *J. Chem. Soc.*, 1183–1190.

Ashwell, G., 1957. Colorimetric analysis of sugars. *In:* Methods in Enzymology, edited by S. P. Colowick and N. O. Kaplan, Academic Press, New York.

Bailey, J. M., and W. J. Whelan, 1961. Physical properties of starch. I. Relationship between iodine stain and chain length. *J. Biol. Chem.*, **236**: 969–973.

Barber, A. A., W. W. Harris and G. M. Padilla, 1965. Studies of native glycogen isolated from synchronized *Tetrahymena pyriformis* (HSM). *J. Cell Biol.*, **27**: 281–292.

Barbieri, F. D., and J. A. Gil, 1962. Utilización del glucógeno en huevos en desarrollo de *Bufo arenarum*. *Arch. Bioq. Quim. Farm.—Tucumán*, **10**: 41–47.

Barbieri, F. D., and H. Salomón, 1963. Some experimental studies on glycogen and lactic acid in the developing eggs of *Bufo arenarum*. *Acta Embryol. Morphol. Exper.*, **6**: 304–310.

Barth, L. G., 1946. Studies on the metabolism of development. *J. Exp. Zool.*, **103**: 463–486.

Barth, L. G., and L. J. Barth, 1954. The Energetics of Development. Columbia University Press, New York.

Boell, E. J., 1955. Energy exchange and enzyme development during embryogenesis. *In:* Analysis of Development, edited by B. H. Willier, P. A. Weiss and V. Hamburger, W. B. Saunders Co., Philadelphia.

Brachet, J., 1934. Étude du métabolisme de l'oeuf de grenouille (*Rana fusca*) au cours du développement. I. La respiration et la glycolyse, de la segmentation à l'éclosion. *Arch. Biol.*, **45**: 611–727.

Brachet, J., 1960. The Biochemistry of Development. Pergamon Press, London.

Brachet, J., and J. Needham, 1935. Étude du métabolisme de l'oeuf de grenouille (*Rana fusca*) au cours du développement. IV. La teneur en glycogene de l'oeuf de la ségmentation à l'éclosion. *Arch. Biol.*, **46**: 821–835.

Cohen, A. I., 1954. Studies on glycolysis during the early development of the *Rana pipiens* embryo. *Physiol. Zoöl.*, **27**: 128–141.

Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers and F. Smith, 1956. Colorimetric method for determination of sugars and related substances. *Anal. Chem.*, **28**: 350–356.

Fischer, E. H., and E. A. Stein, 1960. α-Amylases. *In:* The Enzymes, edited by P. D. Boyer, H. Lardy and K. Myrbäck, Academic Press, New York.

French, D., 1960. β-Amylases. *In:* The Enzymes, edited by P. D. Boyer, H. Lardy and K. Myrbäck, Academic Press, New York.

Gregg, J. R., 1948. Carbohydrate metabolism of normal and of hybrid amphibian embryos. *J. Exp. Zool.*, **109**: 119–134.

Gregg, J. R., and S. Løvtrup, 1950. Biochemical gradients in the axolotl gastrula. *C. R. Lab. Carlsberg, Ser. Chim.*, **27**: 307–324.

Gregg, J. R., J. J. MacIsaac and M. A. Parker, 1964. Anaerobic glycolysis in amphibian development. Homogenates. *Biol. Bull.*, **127**: 256–270.

Heatley, N. G., and P. E. Lindahl, 1937. Studies on the nature of the amphibian organization center. 5. The distribution and nature of glycogen in the amphibian embryo. *Proc. Roy. Soc. London, Ser. B.*, **122**: 395–402.

Jaeger, L., 1945. Glycogen utilization by the amphibian gastrula in relation to invagination and induction. *J. Cell. Comp. Physiol.*, **25**: 97–120.

KRISMAN, C. R., 1962. A method for the colorimetric estimation of glycogen with iodine. *Anal. Biochem.,* **4**: 17–23.

LEGNAME, A. H., AND F. D. BARBIERI, 1962. El metabolismo respiratorio en el desarrollo inicial de *Bufo arenarum. Arch. Bioq. Quim. Farm.—Tucumán,* **10**: 31–39.

LØVTRUP, S., 1955. Chemical differentiation during amphibian embryogenesis. *C. R. Lab. Carlsberg, Sér. Chim.,* **29**: 261–314.

MANNERS, D. J., 1957. The molecular structure of glycogens. *Advances in Carbohydrate Chemistry,* **12**: 261–298.

MORDOH, J., C. R. KRISMAN AND L. F. LELOIR, 1966. Further studies on high molecular weight liver glycogen. *Arch. Biochem. Biophys.,* **113**: 265–272.

OLAVARRÍA, J. M., AND H. N. TORRES, 1962. Mechanism of action of liver α-amylase. *J. Biol. Chem.,* **237**: 1746–1751.

PISANÓ, A., 1956. Método para mantener la hipófisis de anfibio fisiológicamente "in vitro." *Arch. Farm. Bioq.—Tucumán,* **7**: 387–392.

RAVEN, CH. P., 1935. Experimentelle Untersuchungen über den Glycogenstoffwechsel des Organisationszentrums in der Amphibiengastrula. II. *Proc. Kon. Ned. Akad. v. Wetensch., Amsterdam,* **38**: 1107–1109.

SCOLLO LAVIZZARI, G., 1956. Il comportamento delle alfa e beta amilasi durante lo sviluppo embrionale e larvale di *Bufo vulgaris. Ric. Scient.,* **26**: 2146–2152.

SCHLAMOWITZ, M., 1951. On the nature of rabbit liver glycogen. II. Iodine absorption spectrum. *J. Biol. Chem.,* **190**: 519–527.

SPIEGELMAN, S., AND H. B. STEINBACH, 1945. Substrate-enzyme orientation during embryonic development. *Biol. Bull.,* **88**: 254–268.

STAUDINGER, H., 1948. Über natürliche Glykogene. *Makromol. Chem.,* **2**: 88–108.

STETTEN, D. W., AND M. R. STETTEN, 1960. Glycogen metabolism. *Physiol. Revs.,* **40**: 505–534.

STETTEN, M. R., H. M. KATZEN AND D. STETTEN, 1956. Metabolic inhomogeneity of glycogen as a function of molecular weight. *J. Biol. Chem.,* **222**: 587–599.

SWANSON, M. A., 1948. Studies on the structure of polysaccharides. IV. Relation of the iodine color to the structure. *J. Biol. Chem.,* **172**: 825–837.

THOMA, J. A., AND D. FRENCH, 1960. The starch-iodine-iodide interaction. Part 1. Spectrophotometric investigations. *J. Amer. Chem. Soc.,* **82**: 4144–4147.

URBANI, E., 1962. Comparative biochemical studies on amphibian and invertebrate development. *In*: Advances in Morphogenesis, edited by A. Abercrombie and J. Brachet, Academic Press, New York.

URBANI, E., AND G. SCOLLO LAVIZZARI, 1955. Studio delle amilasi nello sviluppo embrionale e larvale di *Bufo vulgaris. Ric. Scient.,* **25**: 1151–1159.

WALLACE, R. A., 1961. Enzymatic patterns in the developing frog embryo. *Develop. Biol.,* **3**: 486–515.

WOERDEMANN, M. W., 1933. Ueber den Glycogenstoffwechsel des Organisationszentrums in der Amphibiengastrula. *Proc. Kon. Ned. Akad. v. Wetensch., Amsterdam,* **36**: 189–193.

ASSOCIATION-FORMATION BETWEEN PHOTIC AND SUBTLE GEOPHYSICAL STIMULUS PATTERNS—A NEW BIOLOGICAL CONCEPT[1]

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Living systems have recently been demonstrated to be able to distinguish among strengths and vector directions of magnetic (Brown, 1962a; Palmer, 1963; Picton, 1966), electrostatic (Brown, 1962b), and gamma radiation fields (Brown, 1963; Brown and Park, 1964) of the order of magnitude of those of their natural terrestrial environment. Such responsiveness and a number of its properties have been assayed by quantitative studies of orientational tendencies of organisms as diverse as *Paramecium, Volvox,* planarians, snails, and fruit flies. Demonstrations of such properties as (a) that the maximum capacity of mud-snails to resolve direction of a horizontal magnetic vector occurs at the strength of the local natural one (Brown, Barnwell and Webb, 1964), (b) that effects of brief exposures to magnetic fields deviating slightly from the earth's may persist for many minutes following restoration of the natural field (Brown, Barnwell and Webb, 1964; Brown and Park, 1965a), (c) that a sense of geographic direction in the absence of all obvious environmental cues can be duplicated by a response to experimental horizontal magnetic vectors (Brown, 1962a; Brown, Webb and Barnwell, 1964), and (d) that monthly (lunar day) periodisms in behavior can be abruptly phase-shifted by altering direction of the horizontal magnetic vector (Brown and Park, 1965b), suggested that response to geomagnetism might play some normal role in the lives of the organisms.

In addition, numerous unpublished observations during extensive studies with planarians and mud-snails collectively suggested that organisms possessed some kind of "memory" for geographic directions which did not depend upon any obvious cues. The hypothesis was suggested that the living systems might form associations between their ambient fields of obvious factors and the concurrent pervasive three-dimensional complex of electromagnetic forces of their environment. To test this hypothesis the following series of simple experiments were designed and conducted.

Materials and Methods

About 20,000 *Dugesia dorotocephala* were collected in the field on one day in September, 1965, and were maintained in the laboratory for the duration of the study. They were kept in enameled steel containers with aluminum covers that excluded nearly completely the laboratory illumination. The containers were set in running tap water whose temperature ranged systematically through the year from about 19° C. in July to about 5° C. in February. The worms were exposed to the diffuse laboratory illumination for about 2 hours twice a week while they were fed beef liver.

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[1] This study was aided by a contract with the Office of Naval Research (1228-30) and by a grant from the National Science Foundation (GB 3481).

Employing apparatus and methods that have been described in some detail in earlier reports (Brown, 1962a, 1963) on planarian orientational tendencies, samples of the worms taken from the stock supply were assayed usually between 9 and 11 AM in each of four types of experimental series. Unvarying were the worms' field of illumination and all other obvious orienting influences in the worms' environment.

The fixed light field for the worms consisted of (1) a point-source directly above the origin of the one-inch test course and hence non-orienting, (2) a horizontal point-source directly behind the initially directed worm, and (3) a horizontal point-source at right-angles to the right of the initial path direction. In this 3-light field the mean path of the test worms always veered to the left, reflecting their negative phototaxis. The degree of the turning was quantified as the angular deviation from straight forward (0°) of the worms' path at the end of a 1-inch free run. The variables were geographic orientation of the whole apparatus and hence initial orientation of the worms, and time. In each of the four types of experimental series, five or six worms were placed in the apparatus at the beginning of the series and were test-run repeatedly until the the series was finished. Then these worms were permanently discarded.

Series IA comprised determining in immediate succession the mean of each of three 15-path samples, requiring about 5 minutes for each sample, for initially North-directed flatworms and then rotating the apparatus with its contained worms *clockwise* by 180° to South and assaying again the mean paths for three immediately following 15-path samples. Series IB involved the same procedure and sequence except that the apparatus was rotated 180° *counterclockwise.* Series IA and IB were carried out first with equal frequency, and fresh worms were always used for the second.

Series IIA involved a fully parallel procedure to IA, except that the worms were assayed first while South-directed followed by 180° clockwise rotation of the apparatus to North and reassay of the worms. Series IIB was like IIA except that the direction of apparatus rotation was counterclockwise.

All experimenters worked with equal frequency with both Series I and Series II. Two different, identically constructed, orientation apparatuses were employed and usually on any given day both a Series I and a Series II were being conducted concurrently. The study was extended rather uniformly over a period of 10 synodic months—from October 8, 1965, through July 29, 1966. By this means all uncontrolled geophysical variables were essentially randomized. The number of series observed for each calendar month was as follows:

		Series I N → S	Series II S → N
1965	1 October (8)	13	11
	2 November	19	16
	3 December	20	20
1966	4 January	26	24
	5 February	28	28
	6 March	23	24
	7 April	21	20
	8 May	20	21
	9 June	16	17
	10 July (29)	21	19
		207	200

The mean turning response was calculated for the three 15-path samples for the initial direction, North or South. Then the mean turning was determined for the first, second and third 15-path samples immediately following the 180° directional change. The values following clockwise rotations were computed separately from those following the counterclockwise ones.

The data for each of the four series for the 10 months were next reduced to mean turning for each day of the synodic month from full moon minus 15 days to full moon plus 15 days, and three-day moving means for these monthly variations were calculated. Such a moving mean was employed to provide a more dependable indication of any systematic variation related to moon phase since each value could be the mean of a sample of 18 to 21 days of data instead of only 5 to 8, and at the same time, appropriately less emphasis would be accorded single monthly-day means which by chance had been based upon a smaller number of days.

RESULTS

In Figure 1 are depicted the mean monthly variations obtained for the worms *initially directed Northward* for the two independent series conducted consecutively on a given day, together with the mean monthly variations of the same two

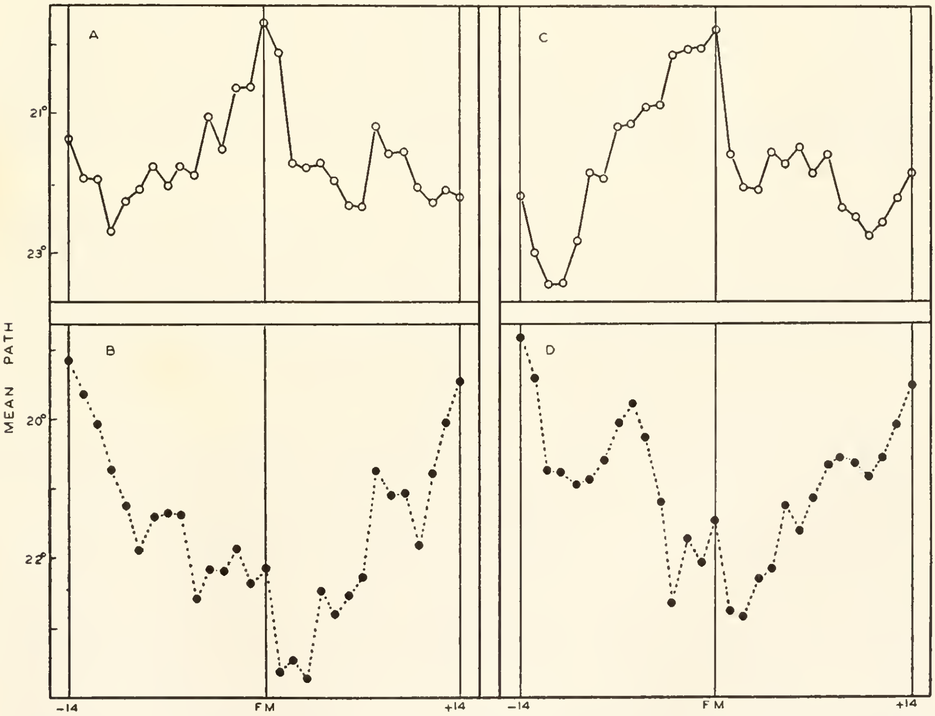

FIGURE 1. A. Mean monthly variation in path of *Dugesia* initially directed *North* with orienting light sources behind and to the right of the worms during a 15-minute assay period. B. Same, immediately after 180° clockwise rotation of the whole apparatus to South. C and D. Repeat of same except with counterclockwise rotation. Ordinate: Degrees of turn to left. Abscissa: Days relative to full moon.

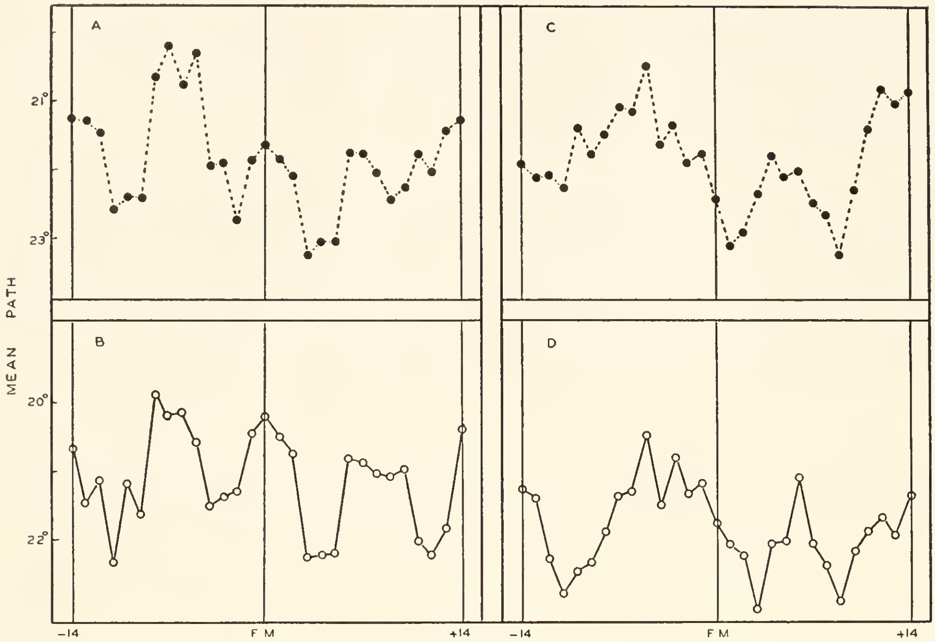

FIGURE 2. A. Mean monthly variation in path of *Dugesia* initially directed *South* with orienting light sources behind and to the right of the worms during a 15-minute assay period. B. Same, immediately after 180° clockwise rotation of the whole apparatus to North. C and D. Repeat of same, except with counterclockwise rotation.

worm-samples after rotation of the apparatus to South, clockwise and counterclockwise. Generally similar to one another are the two independently obtained monthly patterns of variation of the worms while Northbound (Fig. 1, A and C). Quite different, however, are the monthly patterns of variation following the 180° rotation. The two independently derived patterns for these South-directed worms (Fig. 1, B and D) resemble one another rather well despite the opposite directions of the preceding rotation from North to South.

In Figure 2 are shown the comparable monthly patterns for the worms *initially directed Southward* and thereafter rotated to North. Notable in this figure are several things. First, the two independently determined monthly patterns for the South-directed worms (Fig. 2, A and C) are both quite different from the patterns for the South-directed worms immediately following rotation from North (Fig. 1, B and D). They also show some striking differences between them, though for both the maximum for right turning occurs 5 to 7 days *before* full moon and the minimum occurs *after* full moon. But equally evident is an apparent strong tendency for the monthly pattern of variation of the worms after rotation to North to repeat, in general, the same monthly pattern shown by them when previously South-directed (Fig. 2, B and D). Again, as in Series IA and IB, the character of the pattern after rotation appeared independent of the direction of the rotation.

In Figure 3 are plotted the mean monthly patterns, with about a quarter of a cycle repeated, and now centered on new moon. The data for the two series initially North-directed have been averaged together, neglecting direction of rotation, as have also the two initially South-directed ones. In Figure 3A the monthly variation of the South-directed worms following initial North-direction has been temporally displaced by 180°. Evident by inspection is the fact that the worms rotated to South, after North, have a monthly variation of closely the same form

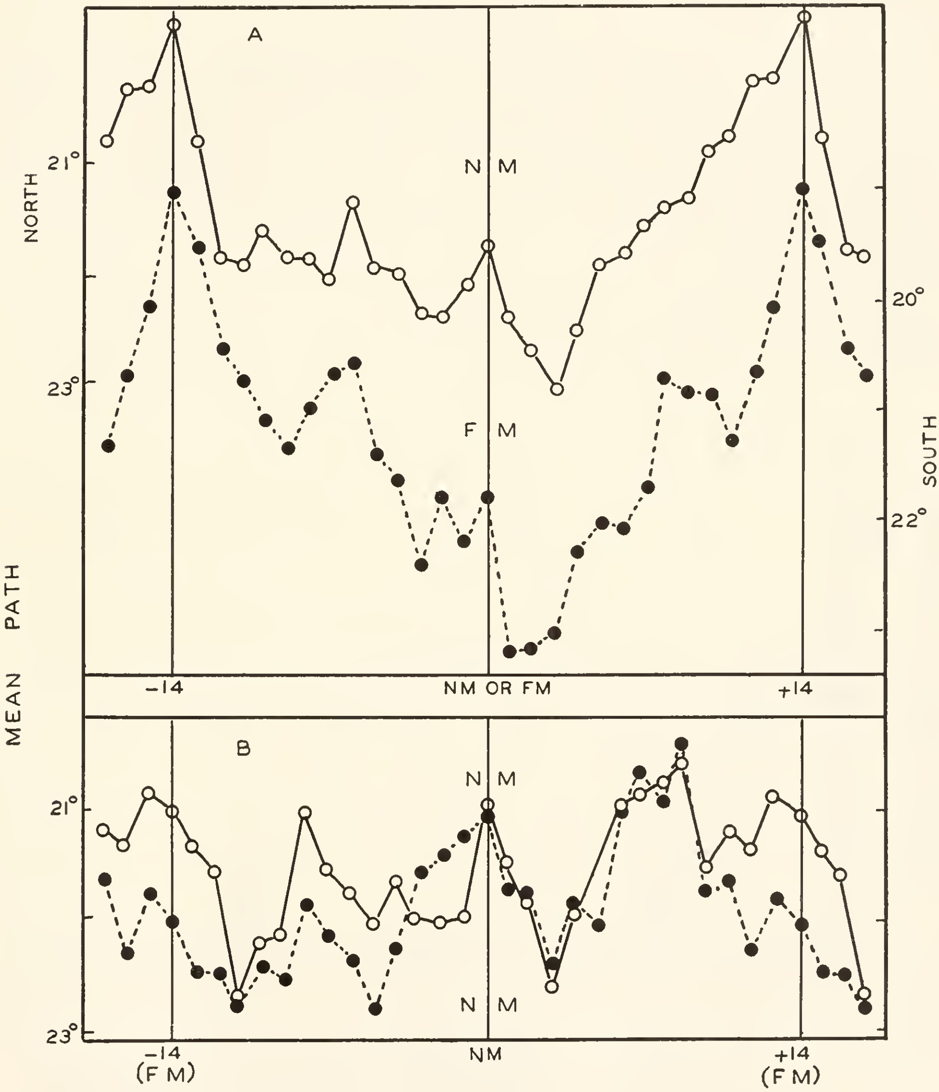

Figure 3. A. Combined values for clockwise and counterclockwise rotated worms first North-directed (open circles) and secondly South-directed (dots). The South-directed lunar monthly relationship has been 180° phase-shifted relative to the North-directed. B. The comparable combined data for the worms first South-directed (dots) and then North-directed (open circles).

as that which was present when initially North-directed but has become temporally displaced by 180° and has a slightly greater amplitude. Again is evident the quite different situation for the initially South-directed worms where the pattern shows a strong tendency to repeat the same form and lunar phase relationships after the 180° rotation to North and even to have essentially the same mean amplitude of variation (Fig. 3B).

Discussion

The form and the phase relations of the monthly variation in turning of the initially North-directed worms resemble closely the pattern that has existed steadily over a continuous five-year investigational period (Brown, 1962a, 1963; Brown and Park, unpublished observations). The variation is overt during Autumn and Winter but in Spring and Summer becomes somewhat obscured by greater variance of the samples. It is, however, readily evident during the latter two seasons as mean monthly cycles of the same gross form and phase relations but of significantly decreased amplitude.

The striking phase-shift of the monthly pattern observed after the initially North-directed worms were rotated to South resembles a comparably altered monthly-pattern phase-shift previously reported to follow an abrupt experimental

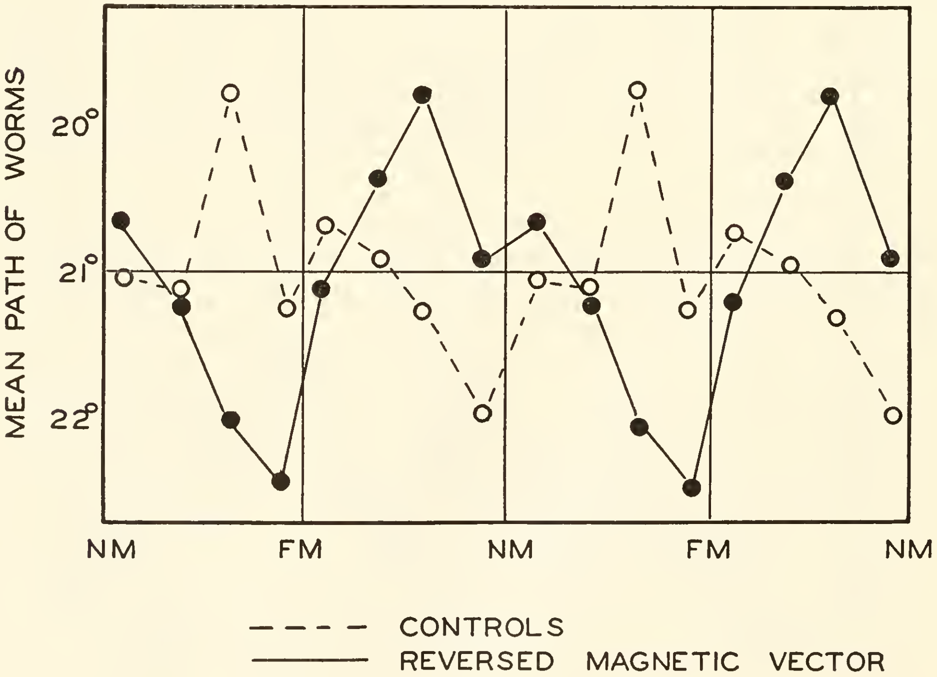

FIGURE 4. The mean monthly variations of North-directed worms during mornings and afternoons in the earth's natural field (open circles) and 30 to 40 minutes after experimental reversal of the horizontal magnetic vector at 0.05 gauss, and, indeed, 15 to 25 minutes after removal of the reversed experimental fields (dots). (Redrawn from Brown and Park, 1965b, to illustrate the 180° temporal phase-shift.)

reversal of the ambient horizontal magnetic vector. When the reversed vector was only about 25% of the strength of the natural geophysical one, the alteration was essentially immediate, but when it was about 25 times the natural field, the alteration was completed only after about 40 minutes of transient states (Brown and Park, 1965b). The latter study had been conducted both mornings and afternoons and the mean monthly cycle-form differed slightly from that obtained for mornings only during the same period. But notable in the results, replotted for comparative purposes in Figure 4, was a very similar 180° temporal shift of the monthly pattern of variation. In neither the present study involving rotation of the apparatus nor the earlier one with reversal of the horizontal vector of magnetism could the results be explained as a simple cycle inversion. In both, the worms appeared to exchange their turning relations with respect to full moon and new moon but at the same time to retain their characteristic asymmetrical cycle-form through what amounted essentially to a 180° cycle-displacement along the temporal axis. In other words, the total monthly pattern was included in the full moon–new moon "exchange." The similarity of the results from these two kinds of experiments, apparatus rotation and magnetic-field rotation, suggests strongly that the chief factor in effecting the results following the 180° rotation of the worms and apparatus in this experiment from North to South is the direction of the ambient horizontal magnetic vector.

The worms are negatively phototactic. While after the rotation of the apparatus from North to South the worms appeared to vary in the strength of their negative phototaxis in a nearly opposite manner, the total explanation cannot be a simple sign-change in phototaxis in view of the detailed asymmetrical pattern participating in the observed alteration.

The worms which were rotated to North into an asymmetrical field with light sources to South and East, after a 15-minute period of residence while South-directed in an asymmetrical field with light sources to North and West, clearly did not respond like worms placed freshly from diffuse illumination into the North-directed apparatus. There must have been an influence of the earlier specific light-magnetic field relationship still persisting after the worms were rotated. And since, comparably, the monthly pattern for South-directed worms with the North and West light sources, after residence for 15 minutes in the asymmetrically lighted field with sources to South and East while North-directed, differed substantially from that of worms placed *initially* in the South-directed field, there must, again, have been a persisting influence of the earlier specific light-magnetic field vector relationship.

It is not known at this time why there should have been the very conspicuous 180° displacement of the characteristic North-directed monthly pattern after the 180° rotation of the worms to South while, on the contrary, there was an equally conspicuous tendency of the worms rotated 180° from South to North to retain not only essentially the same form but also the same phase relationship of their monthly pattern. Hence, while this study indicates the existence of a capacity of the worms to form associations between vector directional components of such overt environmental factors as light on the one hand and such a subtle pervasive geophysical factor as ambient magnetism on the other, and that these associations may persist for at least many minutes, we are still far from a complete understanding of their nature, properties, and biological significance.

From Figure 3A it is seen that after rotation of the apparatus from North to South there appears superimposed on the general 180°-shifted pattern an apparent tendency toward exaggeration of the left-turning behavior over full moon, especially for 1 to 3 days immediately after full moon. This is the time of month of maximum left-turning in the monthly pattern of the worms which are initially South-directed. Correspondingly, in Figure 3B, after rotation of the apparatus from South to North, the major observed difference in otherwise rather similar patterns is an exaggeration of the amount of right-turning over the period of full moon. The last is the normal behavior of the worms when initially North-directed. In brief, these observations suggest that after apparatus-rotation, the mean response pattern for the subsequent period of approximately 15 minutes contains a mixture of two components: (a) a persisting influence of the preceding light-magnetic field vector relationship to which the worms had been exposed, and (b) a characteristic pattern for the worms for that particular geographic direction, other factors equal. Of great interest will be the results of experiments directed toward determining the rate of acquisition of these light-subtle field associations and duration of their persistence.

This study suggests strongly that a living system is able essentially to code an ambient geographic pattern of illumination on a 360° geographic grid of such a subtle geophysical vector field as that of magnetism, and to retain this spatially coded information for at least many minutes. Such a coded geographic "cycle" may provide concurrent alternative, or mutually-supplementing, directional cues, which serve during homing and navigation. The disclosure of such an organismic capacity to code an illumination pattern upon a subtle geophysical variation related to geographic direction provides a basis for postulating that the living system, for any fixed geographic direction, is able to code *temporally* varying information of such an overt factor as illumination on a comparable 360° temporally varying subtle geophysical grid such as that related to the solar day or lunar day. Also supporting such an hypothesis are the gradually accumulating observations that the mechanisms of organismic orientation in time and space possess a common denominator (Brown, 1965). Such an organismic capacity may well serve as a fundamental basis of the phenomena of biological rhythms and clocks and of the apparent clock-dependent astrotaxes.

The "circa" character (not exactly solar-day or lunar-tidal length) of many observed periodisms of animals and plants in unvarying illumination offers no obstacle to an hypothesis that coded, recycling temporal "tapes" are essential, underlying components of the biological clock system, since these odd periodisms may be accounted for as simply a systematic slippage of the cyclic coded patterns along the "tapes" (autophasing).

SUMMARY

1. Evidence is presented that an organism is able to form associations between concurrent ambient vector patterns of light and such a pervasive ambient environmental component as geomagnetism.

2. These associations appear to persist for at least many minutes.

3. Some of the implications of this newly disclosed, extraordinary biological capacity for the still unresolved mechanisms of biological clocks and compasses are discussed.

LITERATURE CITED

Brown, F. A., Jr., 1962a. Responses of the planarian, *Dugesia,* and the protozoan, *Paramecium,* to very weak horizontal magnetic fields. *Biol. Bull.,* **123**: 264–281.

Brown, F. A., Jr., 1962b. Response of the planarian, *Dugesia,* to very weak horizontal electrostatic fields. *Biol. Bull.,* **123**: 282–294.

Brown, F. A., Jr., 1963. An orientational response to very weak gamma radiation. *Biol. Bull.,* **125**: 206–225.

Brown, F. A., Jr., 1965. A unified theory for biological rhythms: Rhythmic duplicity and the genesis of "circa" periodisms: *In:* Circadian Clocks. Ed. J. Aschoff. North Holland Publ. Co., Amsterdam.

Brown, F. A., Jr., and Y. H. Park, 1964. Seasonal variations in sign and strength of gamma-taxis in planarians. *Nature,* **202**: 469–471.

Brown, F. A., Jr., and Y. H. Park, 1965a. Duration of an after-effect in planarians following a reversed horizontal magnetic vector. *Biol. Bull.,* **128**: 347–355.

Brown, F. A., Jr., and Y. H. Park, 1965b. Phase-shifting a lunar rhythm in planarians by altering the horizontal magnetic vector. *Biol. Bull.,* **129**: 79–86.

Brown, F. A., Jr., F. H. Barnwell and H. M. Webb, 1964. Adaptation of the magnetoreceptive mechanism of mud-snails to geomagnetic strength. *Biol. Bull.,* **127**: 221–231.

Brown, F. A., Jr., H. M. Webb and F. H. Barnwell, 1964. A compass directional phenomenon in mud-snails and its relation to magnetism. *Biol. Bull.,* **127**: 206–220.

Palmer, J. D., 1963. Organismic spatial orientation in very weak magnetic fields. *Nature,* **198**: 1061–1062.

Picton, H. D., 1966. Some responses of *Drosophila* to weak magnetic and electrostatic fields. *Nature,* **211**: 303–304.

RESPONSES OF BATS FROM TEMPERATE REGIONS TO CHANGES IN AMBIENT TEMPERATURE

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Although bats in temperate regions are usually thought to migrate to warmer climates or to retreat underground beyond the frost line for the winter, many species commonly encounter freezing temperatures. Big brown bats (*Eptesicus fuscus*) winter in buildings in areas where the outdoor temperature may drop to below −30° C. (Nero, 1959). This bat appears in numbers in caves only during very cold weather, and individuals move to and from their hibernating sites throughout the winter (Mumford, 1958). Pipistrels (*Pipistrellus subflavus*), Indiana bats (*Myotis sodalis*) and little brown bats (*Myotis lucifugus*) all hibernate in caves. The pipistrel is a hardy bat whose numbers present at the place used for hibernation depend upon the severity of the weather (Davis, 1959). Indiana bats enter the caves in mid-autumn, and occasionally die of cold when hibernating too near the entrance. The little brown bat enters hibernation in early autumn, and some individuals retreat into crevices, apparently in response to cold weather. A few bats of this species also succumb to cold during hibernation (Davis and Hitchcock, 1965). The red bat (*Lasiurus borealis*) is a tree-dwelling species which winters in regions where temperatures frequently stay well below freezing for days (Davis and Lidicker, 1956).

As ambient temperature drops below 0° C. a hibernating bat may respond in one of three ways. It may increase its body temperature and arouse from hibernation; it may remain in hibernation and maintain its body temperature by increasing the metabolism sufficiently to compensate for increased heat loss; or it may remain passive during cooling and eventually freeze. Maintenance of a relatively stable body temperature by metabolic compensation has been reported for *Lasiurus borealis* and *Myotis lucifugus* (Reite and Davis, 1966).

The present investigation was undertaken to relate the known differences in the ecology of the five species of bats mentioned above to possible differences in their response to changes in ambient temperature.

MATERIALS AND METHODS

Experimental animals (Table I) were captured in Kentucky. All were obtained from buildings or caves except the red bats, which were netted near certain cave entrances where they regularly appear in late summer and early fall. Upon arrival at the laboratory, the bats were placed in a temperature-controlled room and kept overnight at 5° C. Some were restrained by taping the wings to a wooden block, and a copper-constantan thermocouple connected to a Brown electronic potentiometer was inserted into each bat's rectum. Other bats were left unrestrained with rectal thermocouples taped in place, and still others without any recording device were put into individual jars covered with a screen. The next morning temperature was lowered or increased stepwise by units of 2–10° C. Ambient and rectal temperatures were recorded continuously, and the bats were observed for signs of activity. Electrocardiograms were taken on two or more individuals of each species. The leads were fastened to the skin of the bats with alligator clips, and the signals recorded with a Sanborn amplifier and recording system.

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RESULTS

Ambient temperature of 5° C.

After staying overnight in the room with the temperature control set at 5° C., all specimens appeared to be dormant except for *P. subflavus* and *E. fuscus,* of which species single bats were occasionally found to arouse spontaneously and re-enter the dormant state even when undisturbed. Most unrestrained bats suspended themselves by their feet from the screen covering the jars. *L. borealis* brought the large furred interfemoral membrane up over the body, covering the wings, except at the wrists, and all the ventral surface up to the chin (Fig. 1).

TABLE I

Experimental animals

Species	Number		Dates of collection	Body weights (g.)
	Males	Females		
L. borealis	8	2	Sept.–Oct.	9–13
M. lucifugus	15	10	Sept.–Dec.	7–10
M. sodalis	8	9	Oct.–Dec.	8–10
E. fuscus	8	8	Sept.	19–25
P. subflavus	19	6	Oct.–Dec.	5–6

Rectal temperatures of dormant bats at 5° C. were always less than one degree above ambient. Heart rates varied considerably among species (Table II). Spontaneous and rapid changes in heart rate, not accompanied by noticeable changes in conditions of torpor or rectal temperature, were noted in *E. fuscus* and *P. subflavus.*

Increasing temperature

Stepwise increase in ambient temperature could be performed up to about 10° C. without any change in the appearance of the bats. All seemed to remain dormant. When ambient temperature was changed from 10° C. to 15° C., arousal began in all species except *L. borealis.* In one series of experiments which included 8–12 unrestrained individuals of each of *E. fuscus, P. subflavus, M. lucifugus* and *M. sodalis,* 3–4 bats of each species were active within 60–80 minutes of the increase

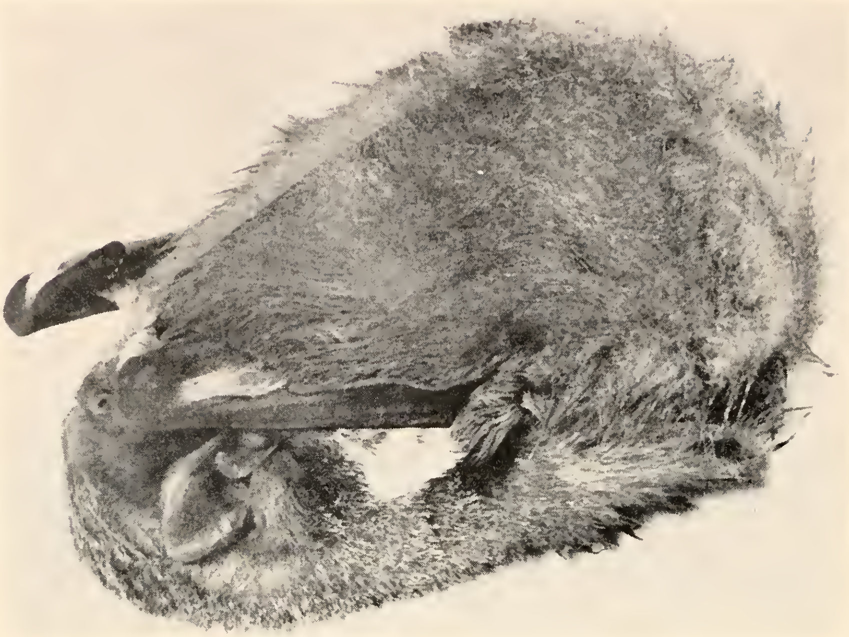

FIGURE 1. Male red bat, *L. borealis,* in dormancy at 5° C.

in temperature. In experiments with *L. borealis* there were no signs of arousal at 15° C. In two individuals of this species taken to higher temperatures, arousal was induced at about 20° C.

During passive warming to 10° C., rectal temperatures remained within 1° C. above ambient. Bats which began to arouse after ambient temperature was raised to 15° C. showed an increase in rectal temperature. In *E. fuscus* the temperature reached 35–38° C. in 1–2 hours.

Heart rates after passive warming of *M. lucifugus, E. fuscus,* and *L. borealis* to 10° C. are given in Table II. Arousal from the dormant state was always accompanied by a rapid increase in heart rate.

TABLE II

Heart rates in dormant bats. Counts were made over several 30–60-sec. periods chosen at random from 6–8 recordings each lasting 5–10 min. and taken at intervals of at least 1 hour

	Ambient temperature (° C.)	Heart rate (beats/min.)
L. borealis	5	10–16
L. borealis	10	16–22
M. lucifugus	5	24–32
M. lucifugus	10	44–56
E. fuscus	5	42–62
E. fuscus	10	64–88
M. sodalis	5	36–62
P. subflavus	5	24–80

Decreasing temperature

Striking differences among species were evident in response to decreasing ambient temperature to 0° C. and below. *E. fuscus* invariably aroused from dormancy and became active within 40–120 minutes. Both restrained and unrestrained individuals were able to remain active for several hours, even at an ambient temperature as low as −5° C. Attempts to make them re-enter dormancy by keeping them overnight at −3° C. to −5° C. were unsuccessful, but this could easily be achieved by changing ambient temperature to 5° C. About half the *P. subflavus* studied aroused following a temperature change from 5° C. to 0° C. None aroused when the temperature was lowered from 5° C. to −5° C. in one step. *M. lucifugus* remained dormant when ambient temperature was lowered to 0° C. Further lowering to −5° C. induced arousal in a few, whereas an abrupt change from 5° C. to −5° C. induced arousal in all bats of this species. *M. sodalis* responded similarly. *L. borealis* remained dormant both during stepwise and abrupt lowering of ambient temperature from 5° C. to −5° C. Abrupt exposure of *L. borealis* to subfreezing temperatures induced an almost instantaneous increase in the rate and depth of respiration.

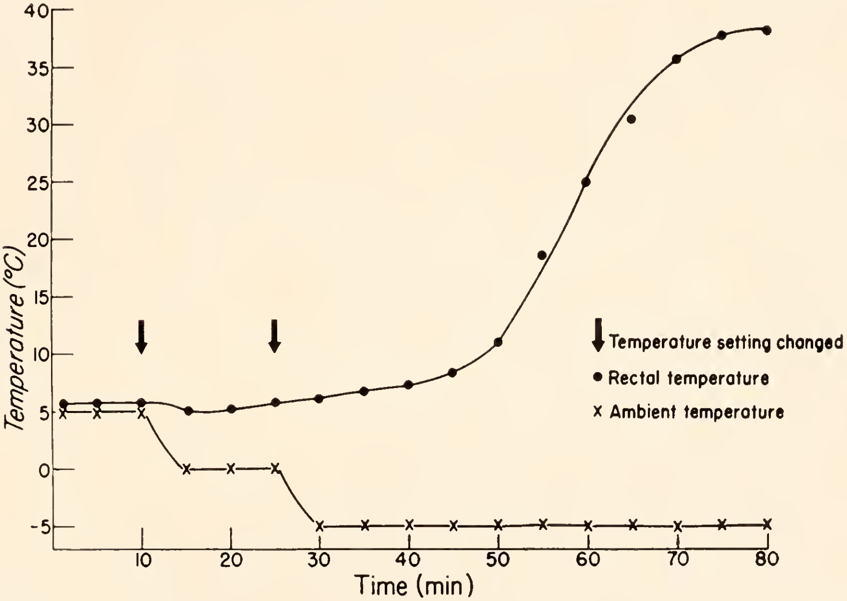

FIGURE 2. Changes in rectal temperature of *E. fuscus* during arousal from dormancy in response to lowering of ambient temperature.

Following a change of ambient temperature from 5° C. to between 0° C. and −5° C., dormant *E. fuscus* showed a slight decrease in rectal temperature followed within about 30 minutes by a rapid increase (Fig. 2). The increase in rectal temperature was accompanied by increasing heart rate. As reported previously (Reite and Davis, 1966), *L. borealis* and *M. lucifugus* show an increase in the difference between rectal and ambient temperature and an increased heart rate when ambient temperature is gradually decreased. A similar response was found in *M. sodalis.*

P. subflavus, tested for response to an abrupt lowering of ambient temperature, showed low rectal temperature even after more than one hour at −5° C. A comparison between unrestrained *P. subflavus* (5 individuals) and *M. lucifugus* (7 individuals) by exposing them to a temperature change from 5° C. to −5° C. revealed a marked difference in their responses. After one hour the rectal temperatures of *P. subflavus* ranged from −1.5° C. to 0° C. Four of the *M. lucifugus* were fully active with rectal temperature ranging from 34° C. to 39° C., and the other three were in various stages of arousal (rectal temperatures 5–14° C.). *P. subflavus* kept at −5° C. for 3–4 hours died.

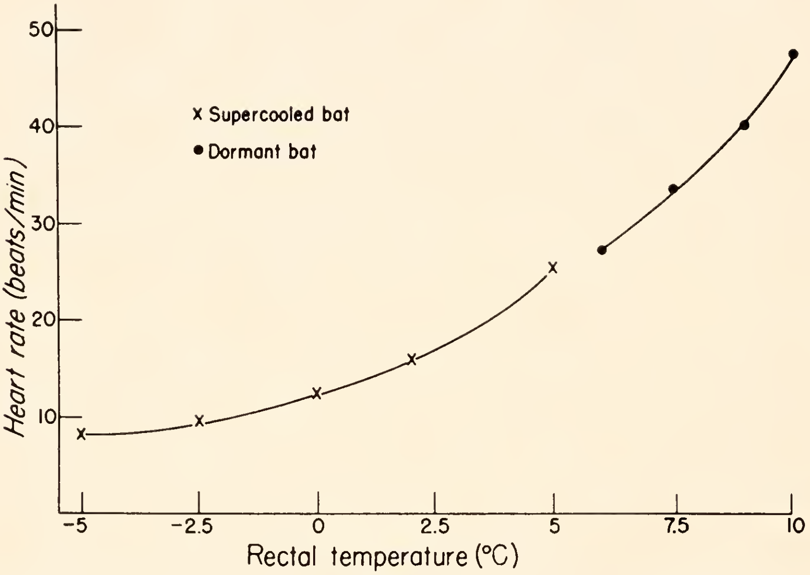

FIGURE 3. Changes in heart rate in *M. lucifugus* during passive warming of a supercooled individual from −5° C. to 5° C. and a dormant one from 5° C. to 10° C.

Supercooling and freezing

Rectal temperatures of restrained dormant *M. lucifugus, M. sodalis* and *L. borealis* kept at −5° C. stayed 1–4° C. above ambient for 2–3 hours, the central body temperature being probably still higher than that of the rectum. During this time, the bats continued breathing and maintained increased heart rate. When kept at −5° C. for longer periods the breathing ceased and rectal temperature dropped to ambient, indicating that the temperature gradients between central parts of the body and the periphery were disappearing. Only bats in this latter condition will be termed supercooled, although local tissue temperatures in bats of the former category were also well below the freezing point of tissue fluids. Spontaneous tissue freezing followed by death occurred in some of the supercooled bats. Whether or not freezing occurred was determined by inspection. In those bats which did not freeze, heart beats continued and rectal temperature remained constant at −5° C. Several bats of each species were removed from the cold room and exposed to room temperatures after having been kept in the supercooled state with ceased breathing for 5–8 hours, while others in the same condition were left in the cold room, where the temperature was then changed to 5° C. We tested all bats for ability to respond to stimuli by forceably opening the eyes and mouth, extending the wings, and probing the bodies with fingers. Unlike the situation during dormancy, the bats lacked muscle tone and did not respond to stimuli. After 8–16 minutes at an ambient temperature of 24° C. the bats which had been removed from the cold room began slight movements of the feet and soon resumed breathing. Within 30–45 minutes they had recovered completely and were capable of normal flight. They were kept overnight in the laboratory, tested again for normal flight, and released in apparently good condition. In the supercooled bats left in the cold room at 5° C., heart rate increased slowly during rewarming (Fig. 3), breathing was resumed, and these bats also recovered completely.

No attempt was made to test survival time of supercooled bats which had ceased breathing. We succeeded in keeping one individual of *M. lucifugus* supercooled at −6.5° C. for half an hour and rewarming it without ice formation. This bat also survived. Occasionally, during exposure to sub-zero temperatures, freezing occurred in peripheral tissues of bats which had not become supercooled. These animals recovered if removed from the cold room before ice formation had taken place in more central parts of the body. However, when spontaneous freezing occurred in already supercooled animals it seemed inevitably to be lethal. Freezing in supercooled bats was accompanied by a rapid rise in body temperature to a level between −0.5° C. and −1° C., and the process could be induced by pricking with a needle. Supercooling to below −5° C. for any length of time seemed difficult, as further lowering of the temperature greatly enhanced the tendency to spontaneous ice formation.

DISCUSSION

Since Eisentraut (1934) published one of the first accounts on the different physiological states of bats with respect to ambient temperature, numerous reports have followed. The literature is covered in a recent review (Stones and Wiebers, 1965).

Hibernating bats have different ways of surviving when ambient temperatures drop below freezing. According to our experiments, *E. fuscus* will awaken, a reaction which also seems to be normal in other hibernators (Hock, 1960). During extremely cold weather it is not unusual for bats of this species to be seen in flight in buildings. Common speculation among students of Chiroptera is that overheating of the hibernating sites by the heating systems of the buildings is the likely cause of arousal. From the present results we believe it is more probable that the hibernating sites become too cold, which causes the bats to move to warmer places. This assumption is supported by the observation that *E. fuscus* undergoes considerable intercave movement at air temperatures below freezing (Mumford, 1958).

L. borealis responds to freezing temperatures by increasing its metabolism enough to maintain its body temperature above a dangerous lower limit (Reite and Davis, 1966). These animals do not hibernate in caves, and it would be to their disadvantage to arouse, since arousal would use far more energy than regulation during dormancy. Red bats also remain dormant when ambient temperature rises. Our observations indicate that whereas other species will arouse at temperatures between 10° C. and 15° C., red bats remain in dormancy up to a temperature of about 20° C. unless handled or otherwise disturbed. This is an important adaptation. These animals are exposed to wide fluctuations in temperature in their natural environment, and cannot afford to become active until it is warm enough to obtain enough food to compensate for loss of stored energy. Thus in winter they do not fly unless it is warm enough for insect flight. Davis and Lidicker (1956) found that red bats became active only on days when temperatures rose to 19° C. or above. Constantine (1958) observed the closely related *L. seminolus* hibernating in their natural environment, and reported that they awoke and flew only when environmental temperatures reached 21° C. Both reports give support to the present findings. *L. borealis* joins other species of bats in swarming at the caves in early fall (Davis, 1964), but never hibernates there. Occasionally, red bats enter rooms in certain caves, cannot find their way out again, so hang up and become dormant. Such bats invariably perish (Myers, 1960), perhaps being unable to arouse spontaneously at cave temperatures. Thus red bats seem to be so adapted to survival outside that they are unable to survive in caves.

The anatomical structure and the behavior of *L. borealis* are better modified for survival at low temperatures than those of any of the other species studied. Except for the ears and parts of the wings, this bat is completely furred. The furred interfemoral membrane and the long tail, in relation to body length, is probably of significance in heat conservation during hibernation when the bat uses the interfemoral membrane to cover the ventral surface (Fig. 1). The short rounded ears may also enable this bat to tolerate exposure to cold better than the other species.

The only bat in which the response was not what might be expected from previous knowledge of its behavior in its natural environment was *P. subflavus*. Since bats of this species move into the caves in numbers only after periods of freezing weather, we would expect them to arouse as temperatures approach and go below freezing. However, our experiments showed that they aroused only in response to moderate lowering of ambient temperature. The reason may be that their small size does not allow them to generate enough heat to exceed the heat loss when ambient temperature is lowered abruptly to −5° C. Even in larger bats the increase in body temperature is slow during the initial steps of arousal. The low tolerance of *P. subflavus* to supercooling makes it reasonable to believe that their natural way of responding is to come out of hibernation. They may survive outside the caves during early moderate cold periods and arouse and move into the caves when the most severe part of the winter is approaching. Folk (1940) has suggested that the first cold periods of winter may indicate to bats the suitability of their resting place for hibernation.

Both Kayser (1940) and Hock (1951) noted increased respiratory exchange in dormant bats exposed to temperatures near 0° C. The justification for considering this as a true thermoregulatory response is supported by the present observations in bats of the species *L. borealis, M. lucifugus* and *M. sodalis,* which established an increased difference between rectal and ambient temperature when the latter was decreased.

If taken as an indicator of metabolism, the heart rate in dormant bats at 5° C. should reflect the relative efficiency or depth of hibernation in the different species. Of the species studied, *L. borealis* may be considered best adapted for hibernation. Those with the highest heart rates (*E. fuscus, M. sodalis* and *P. subflavus*) should be more prone to arousal. This assumption is supported by the finding of spontaneous rapid changes in the heart rate of dormant *E. fuscus* and *P. subflavus,* and also by the observation that individuals from these species occasionally became active and re-entered dormancy even when kept at a stable ambient temperature of 5° C. *M. lucifugus* is intermediate. Whether this bat will respond to a lowering of ambient temperature by increasing its metabolism enough to compensate for increased heat loss (thermoregulation) or by arousal from dormancy, seems to depend on the abruptness and severity of the cold exposure. Seasonal differences may also be present. The heart rate of *M. lucifugus* in dormancy at 5° C. is in the same range as that reported by Johansen and Krog (1959) for the birchmouse, a hibernator of comparable size.

Cooling of bats to about −5° C. without formation of ice in the body is in agreement with previously obtained results (Kalabuchow, 1935). The slow increase in heart rate in supercooled *M. lucifugus* during rewarming from −5° C. to 5° C. (Fig. 3) corresponds fairly well to the rate change found in isolated hearts of this species over that part of the same temperature range where such studies have been performed (Michael and Menaker, 1963). The heart rate in dormant *M. lucifugus* at temperatures of 5–10° C. is also in the same range as that of the isolated heart. These observations suggest that in supercooled bats with ceased breathing and in dormant bats at neutral ambient temperatures (5–10° C.) the heart is not under any neural influence. This is different from the situation in dormant bats of *L. borealis* and *M. lucifugus* exposed to stepwise lowering of ambient temperature from 5° C. In these bats the heart rate increases with decreasing temperature (Reite and Davis, 1966).

Supercooling of bats could be of significance for survival during short term exposure to sub-zero temperatures, a situation which may occur following a change of wind direction at the entrance to a cave used for hibernation. However, supercooling is an unstable condition and must be transient.

We wish to thank Roger W. Barbour for photographing the dormant red bat, and Loren D. Carlson, Eugene C. Crawford and Marion D. Hassell for reading the manuscript.

SUMMARY

1. Responses to upward and downward changes in ambient temperature from 5° C. were studied in dormant bats of the species *E. fuscus, P. subflavus, M. sodalis, M. lucifugus* and *L. borealis.* Rectal temperatures and heart rates were recorded.

2. Except for *L. borealis* which did not arouse until ambient temperature reached about 20° C., all species responded by arousal from dormancy when the temperature was increased to 15° C.

3. The effects of decreasing ambient temperature varied considerably among species. *E. fuscus* invariably aroused from dormancy. *L. borealis* never aroused but showed a thermoregulatory response by increasing its metabolism to compensate

for the increase in heat loss. The responses of the other species depended upon the abruptness of the temperature change. Abrupt lowering of ambient temperature tended to induce arousal in *M. lucifugus* and *M. sodalis*, whereas these species responded similarly to *L. borealis* when exposed to gradually decreasing temperature. *P. subflavus* usually aroused in response to a gradual decrease in ambient temperature, but seemed unable to arouse in response to abrupt lowering of temperature.

4. Bats of the species *L. borealis*, *M. lucifugus* and *M. sodalis* supercooled to −5° C. showed cessation of breathing, but slow heart beats continued for several hours. Passive rewarming was necessary for survival.

5. Many of the known differences in the ecology of the studied species of bats are reflected as differences in their response to changes in ambient temperature in the laboratory.

LITERATURE CITED

Constantine, D. G., 1958. Ecological observations on lasiurine bats in Georgia. *J. Mammal.*, **39**: 64–70.

Davis, W. H., 1959. Disproportionate sex ratios in hibernating bats. *J. Mammal.*, **40**: 16–19.

Davis, W. H., 1964. Fall swarming of bats at Kentucky caves. *Bull. Natl. Speleolog. Soc.*, **26**: 82–83.

Davis, W. H., and H. B. Hitchcock, 1965. Biology and migration of the bat, *Myotis lucifugus*, in New England. *J. Mammal.*, **46**: 296–313.

Davis, W. H., and W. Z. Lidicker, Jr., 1956. Winter range of the red bat, *Lasiurus borealis*. *J. Mammal.*, **37**: 280–281.

Eisentraut, M., 1934. Der Winterschlaf der Fledermäuse mit besonderer Berücksichtigung der Wärmeregulation. *Z. Morphol. Ökol. Tiere*, **29**: 231–267.

Folk, G. E., Jr., 1940. Shift of population among hibernating bats. *J. Mammal.*, **21**: 306–315.

Hock, R. J., 1951. The metabolic rates and body temperatures of bats. *Biol. Bull.*, **101**: 289–299.

Hock, R. J., 1960. Seasonal variations in physiologic functions of arctic ground squirrels and black bears. *In:* Mammalian Hibernation. Ed. by C. P. Lyman & A. R. Dawe. *Bull. Mus. Comp. Zool.*, **124**: 155–169.

Johansen, K., and J. Krog, 1959. Diurnal body temperature variations and hibernation in the birchmouse, *Sicista betulina*. *Amer. J. Physiol.*, **196**: 1200–1204.

Kalabuchow, N. I., 1935. Anabiose bei Wirbeltieren und Insekten bei Temperaturen unter 0°. *Zool. Jahrb., Abt. allg. Zool. Physiol.*, **55**: 47–64.

Kayser, Ch., 1940. Les échanges respiratoires des hibernants à l'état de sommeil hivernal. *Ann. Physiol. Physicochim. Biol.*, **16**: 127–221.

Michael, C. R., and M. Menaker, 1963. The effect of temperature on the isolated heart of the bat, *Myotis lucifugus*. *J. Cell. Comp. Physiol.*, **62**: 355–358.

Mumford, R. E., 1958. Population turnover in wintering bats in Indiana. *J. Mammal.*, **39**: 253–261.

Myers, R. F., 1960. *Lasiurus* from Missouri caves. *J. Mammal.*, **41**: 114–117.

Nero, R. W., 1959. Winter records of bats in Saskatchewan. *Blue Jay*, **17**: 78.

Reite, O. B., and W. H. Davis, 1966. Thermoregulation in bats exposed to low ambient temperatures. *Proc. Soc. Exp. Biol. Med.*, **121**: 1212–1215.

Stones, R. C., and J. E. Wiebers, 1965. A review of temperature regulation in bats (Chiroptera). *Am. Midl. Naturalist*, **74**: 155–167.

VESICULATED AXONS IN HAEMAL VESSELS OF AN HOLOTHURIAN, CUCUMARIA FRONDOSA

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There is only fragmentary evidence of neurosecretory activity in echinoderms (Fontaine, 1962; Bullock and Horridge, 1965). A primitive haemal system occurs as a rete in the sea cucumber, *Cucumaria.* In this rete we have found nerve strands containing large dense-cored vesicles in axons which are distributed to the non-striated muscle of the vessel wall.

Specimens were collected from Frenchman Bay, Maine, and kept in running sea water at 15° C. for a few days. Segments of the haemal rete were ligated to prevent contraction, excised and fixed for electron microscopy. Fixation in a 3.5% glutaraldehyde in phosphate buffer was followed by treatment with 1% osmium tetroxide. Other specimens were fixed in 1% osmium tetroxide with 0.3 *M* sucrose in the fixative, followed by 10% formalin. Tissues were embedded in epoxy resin and sections stained with uranyl acetate and lead citrate.

The wall of the haemal vessel has three layers. The outer layer consists of coelomic epithelial cells, nerve strands and non-striated muscle cells. The intermediate layer consists of a thick, distinctly filamentous basal lamina adjacent to the muscle fibers and a deeper connective tissue consisting of a mucoid matrix containing fibers with a periodicity of 640 Å. The inner layer consists of more or less contiguous cells with processes embedded in the fibrous layer, constituting an endothelium. The "endothelial" cells have fine structural features similar to those of fibroblasts in higher forms.

In the outer layer the coelomic epithelial cells have a columnar peripheral portion containing large oil droplets and, at the level of the nucleus, a prominent Golgi region. The basal portion of these cells extends as two or more tapering processes which pass between the fibrous portions of the muscle cells to reach the basal lamina (Figs. 1, 6, 7 F). In these processes masses of fine parallel filaments fill the terminal portions. Adjacent to the basal lamina the fibrous portions of the muscle cells are oriented in the plane of the basal lamina. Most of the fibers are circumferential but a few are longitudinal. There are cytoplasmic processes from the muscle cell extending into the basal lamina. The nuclei and most of the cytoplasm of the muscle cells are found peripherally among the processes of the coelomic epithelial cells but most of the mitochondria are adjacent to the myofilaments. Rather narrow strands of cytoplasm may connect the nuclear and fibrillar portions of the muscle cells (Fig. 1).

Passing between the numerous processes of the coelomic and muscle cells are groups of axons forming nerve strands (Fig. 1 N). At bifurcations of the rete the strands of axons apparently cross each other (Fig. 4). Nerve strands three to five microns in diameter are common in the wall of the haemal rete (Figs. 2, 3). From these strands single axons are distributed to the fibrous portions of the muscle cells (Fig. 6). Individual axons vary considerably in cross-sectional area (Fig. 4) and there are frequent expansions which may be rather empty or containing accumulations of mitochondria, lipid and dense-cored vesicles.

Within the axons microtubules are preserved after glutaraldehyde fixation (Fig. 3) and are quite uniformly 260 Å in diameter. A small amount of granular lipid is present. There is a variety of sizes of vesicles in the axoplasm and rows of large (0.2 to 0.3 micron) membrane-bounded bodies with a clear space surrounding a

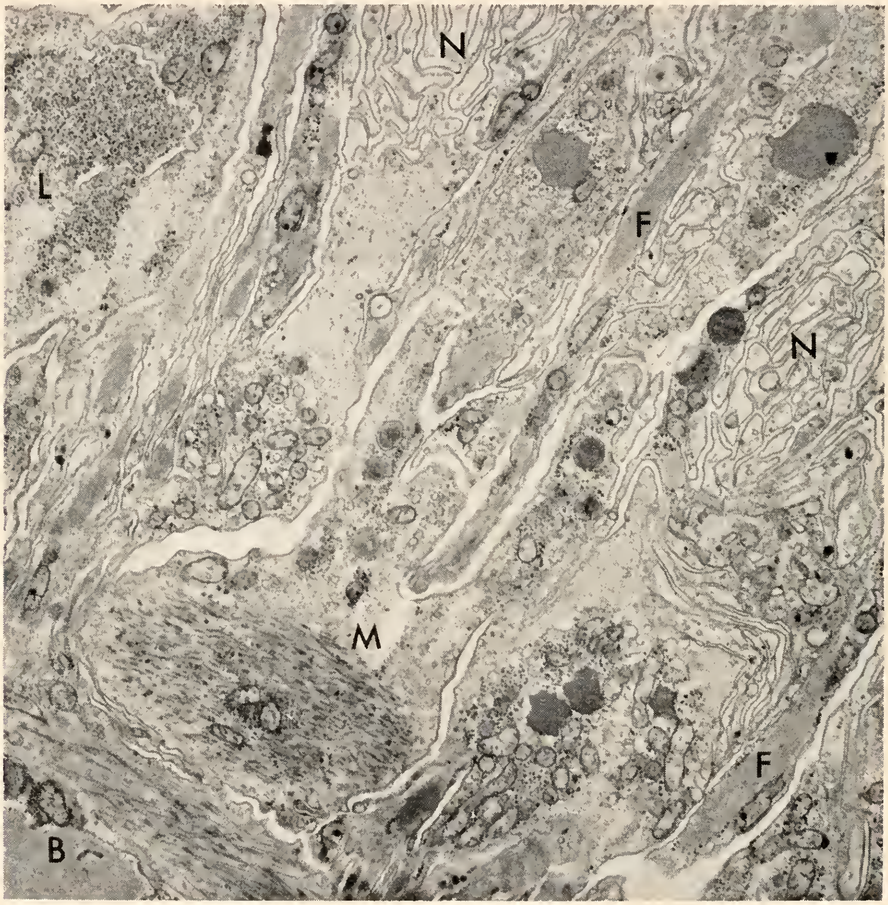
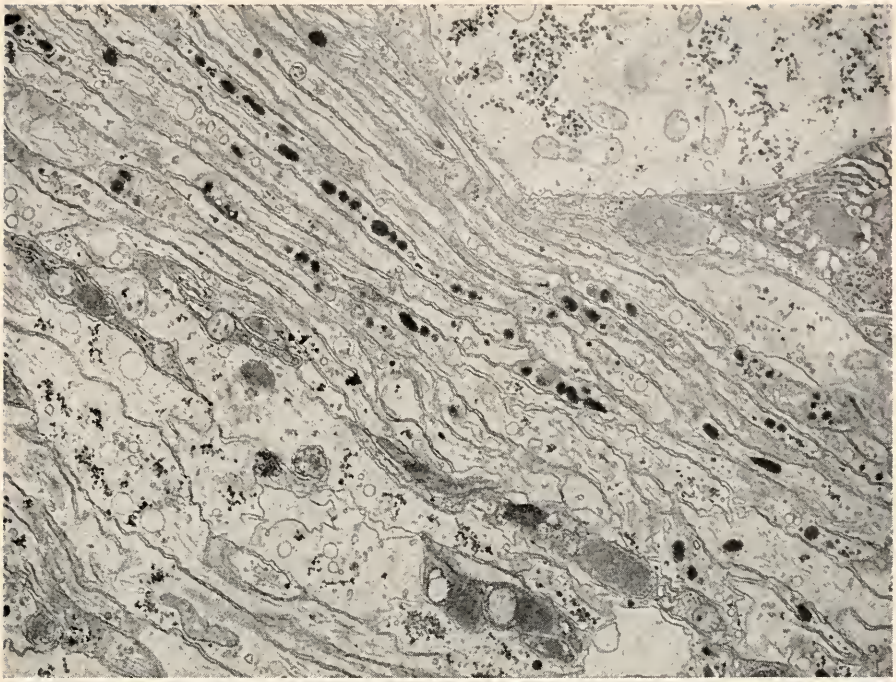


FIGURE I. Cross-section of the inner portion of the outer layer of the wall of the haemal vessel. Basal lamina, B, at lower left corner and muscle fibers, M, adjacent. The slender dense cell processes extending diagonally across the figure are extensions of coelomic epithelial cells containing filamentous masses, F, and a few mitochondria and lipid granules. A cell with lipid granules, L, is at upper left. In one muscle cell, M, sections of four mitochondria are located among the muscle fibrils and have associated lipid granules. The larger groups of mitochondria in adjacent cells are in the cytoplasmic portions of other muscle cells. Cross-sections of parts of two nerve strands, N, are evident midway in the upper and right hand edges of the figure. Osmic fixation, 7900 ×.



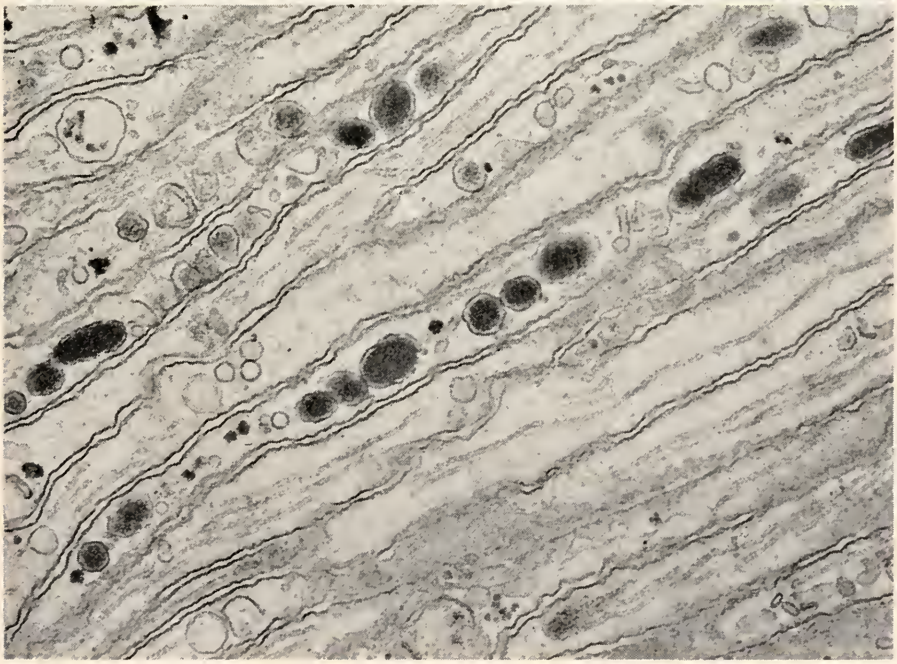


FIGURE 2. Longitudinal section of a nerve strand with several axons running from upper left to lower right. Within the axons are rows of dense vesicles. Glutaraldehyde fixation, 8500 ×.

FIGURE 3. Parallel axons in a nerve strand containing microtubules 260 Å in diameter and membrane-bounded dense vesicles 0.1 to 0.3 micron in diameter. The individual axons approximate 0.5 micron in width. Glutaraldehyde fixation, 27,000 ×.

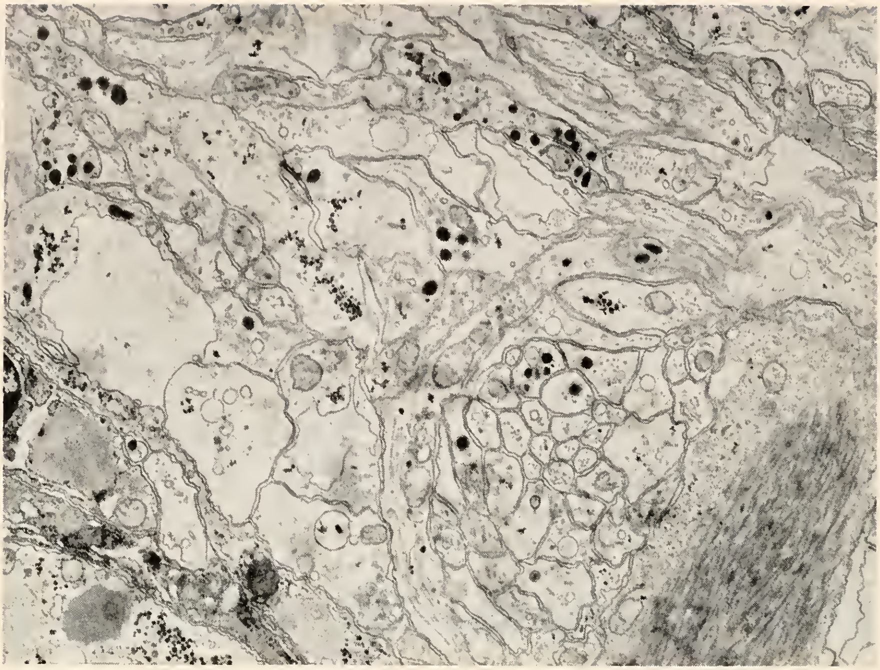


FIGURE 4. Section through a plexus of axons illustrating variations in diameter. Diameters at lower right range from 0.4 to 1.5 microns. Other axons may be identified by the presence of microtubules and specific dense vesicles. The small dense granules in axons are lipid. Glutaraldehyde fixation, 9200 ×.

dense core. These specific vesicles are often elongate and the density of the core varies. In less dense cores the contents can be seen to consist of aggregates of smaller granules or dense vesicles.

No ganglia have been found in the wall of the rete and only occasional single nerve cell bodies along the nerve strands. These cell bodies are distinguished by a folded nucleus, a dilated endoplasmic reticulum which has more ribosomes associated with it in the perinuclear region than in peripheral zones, relatively few mitochondria, a Golgi region, microtubules in the cell processes and in some cases large numbers of vesicles. In one such cell (Fig. 5) the cytoplasm is filled with a variety of heavy-walled vesicles with granular and vesicular contents. In Figure 5 these vesicles are in close association with the Golgi region and give the impression of arising from it. The contents of these vesicles are much less dense than seen in the vesicles found in the axons and some look like multivesiculate bodies. In general, however, the contents are more heterogeneous than seen in multivesiculate bodies and in the elongate forms the contents form denser aggregates. In other cell bodies the dense-cored vesicles have been fewer in number but uniformly more dense, with only a few showing a multivesiculate appearance. Wherever found, the densest particles frequently give some evidence of aggregated composition. It is uncertain whether these bodies are the precursors of those found in the axons but both the heterogeneous and the dense-cored vesicles are confined to the nerve cell body and axons. No similar vesicles have been found in coelomic epithelial cells, and in hundreds of sections containing muscle cells we have found only a few similar granules in muscle cell cytoplasm.

Sections of cell processes distant from the cell body have been found containing much more uniform populations of a hundred or more dense-cored vesicles. Dilations of the axons in the nerve strands also show accumulations of several vesicles and a few mitochondria and lipid granules.

Axons leaving the nerve strand are often about 0.6 micron in diameter and taper gradually to 0.2 to 0.3 micron at their terminations at the muscle cells. Single dense vesicles may fill the cross-section of the axon termination.

At the muscle cell surface the axons terminate as slightly flattened processes with a distinct intercellular space between the membranes of muscle and nerve cells.

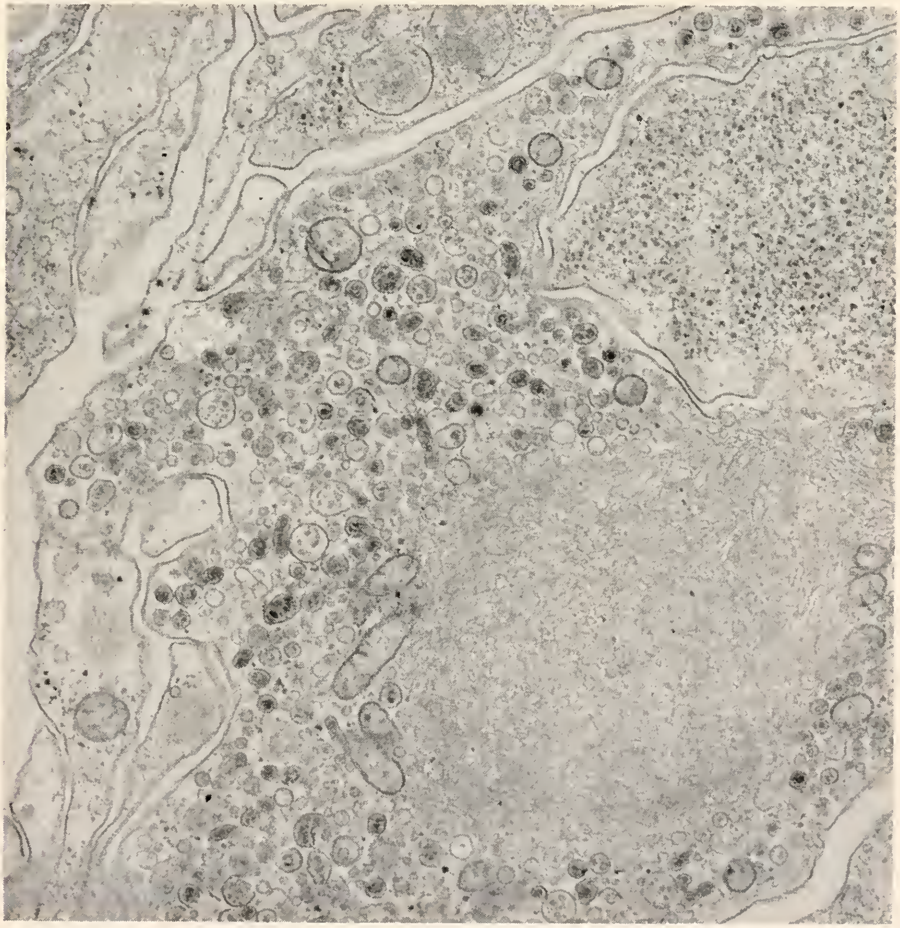


FIGURE 5. Portion of a cell body with a tangential section of the Golgi region surrounded by vesicles of a variety of sizes including some multivesiculate forms. The internal vesicles are of differing densities and degrees of aggregation. Glutaraldehyde fixation, 16,000 ×.

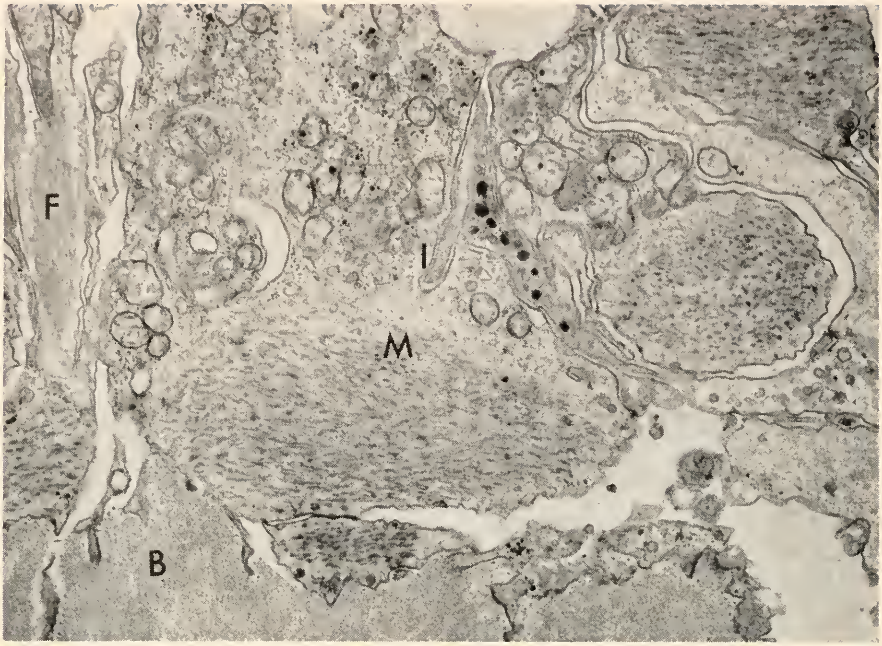


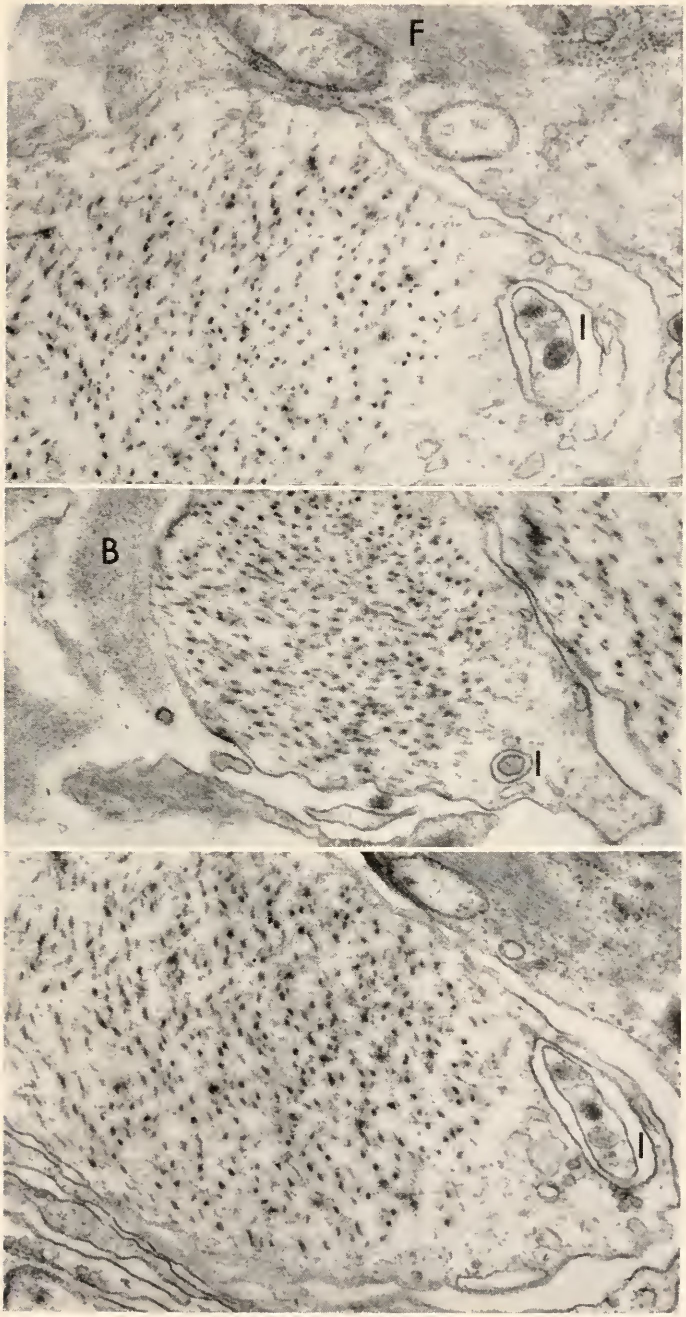
FIGURE 6. Section of a muscle fiber, M, with axon terminals. One axon contains the specific dense vesicles and adjoins the muscle cell membrane at the right. Another, without specific granules, inserts into an invagination, I, of the muscle. Osmic fixation, 11,800 ×.

There is no evident membrane specialization in the regions of approximation. Some of the nerve terminations are in channels of the muscle cell surface with overlapping muscle cell processes. Some axons insert into invaginations of the muscle cell surface but always surrounded by distinct extracellular space (Figs. 6, 7, 8, 9).

DISCUSSION

Cytochemical and electron microscopic evidence for the presence of glycogen is negative in these tissues. The large oil droplets of coelomic epithelial cells and of muscle cells have been observed breaking up into small granules of irregular outline as previously described in the respiratory tree (Doyle and McNiell, 1964) and this lipid may substitute for the glycogen of higher forms. In our preparations the small granules of lipid have irregular outlines and appear as densely stained particles in all of the cells of the outer wall of the haemal rete.

The present evidence on the occurrence of large ($0.3\,\mu$) vesicles containing dense aggregates in axons distributed to non-striated muscle cells suggests a possible neurosecretory function in this primitive vascular system. Very little is known of the organization of this part of the nervous system in these organisms. In common with other neurosecretory vesicles the ones present in these axons stain intensely. They appear to arise in the cell body in proximity to the Golgi region. They are distributed along the axons and do not accumulate at the terminals which



FIGURES 7–9. Cross-sections of fibrous portions of muscle cells with tubular invaginations, I, containing axons. Osmic fixation, 35,000 ×.

lack a terminal expansion. The specific vesicles are with rare exceptions confined to the nerve cells and their processes. A specific search for evidence of discharge or transfer of these vesicles has revealed a very few instances of the presence of similar structures in the muscle cell cytoplasm. These very few instances may in fact represent evidence for transfer of vesicles of a transmitter substance but we have no evidence that this is so. In one instance four dense-cored vesicles were seen in the non-fibrous portion of the muscle cell cytoplasm and in another three. In other instances single bodies were observed in the fibrous portion. No evidence has been found of fusion with the cell membrane or discharge from the axon.

Ultrastructural relationships of nerve processes and smooth muscle cells have been described and reviewed recently by Thaemert (1966) while Lever *et al.* (1965) have reported on axon terminals in the arteriolar wall. The occurrence of small specific vesicles has been commonly reported in these studies in higher forms. The close contiguity of nerve and muscle and occurrence of channels in the muscle cell are similar to the relations found in *Cucumaria*. The specific dense vesicles found in *Cucumaria* are much larger structures and their neurosecretory nature remains to be established. Similar structures have been reported in *Hydra* by Lentz and Barrnett (1965).

This work was supported by a grant, GB 3035, from the National Science Foundation.

SUMMARY

Segments of the primitive haemal rete of the holothurian, *Cucumaria*, were fixed in glutaraldehyde and in osmic acid, embedded in epoxy resin, sectioned for electron microscopy and stained with uranyl acetate and lead citrate. Multifibered nerve strands were found among the epithelial cell processes of the wall of the haemal vessels. Individual axons containing large (0.2 to 0.3 micron) membrane-bounded dense-cored vesicles are distributed to the non-striated muscle cells. The vesicles arise in association with the Golgi region of the neurone and large numbers are found in proximal cell processes. The vesicles containing dense aggregates are distributed along the axons, with a few present at the tapered terminal portions at the muscle cell.

LITERATURE CITED

BULLOCK, T. H., AND G. A. HORRIDGE, 1965. Structure and Function in the Nervous Systems of Invertebrates. San Francisco: Freeman.

DOYLE, W. L., AND G. F. MCNIELL, 1964. The fine structure of the respiratory tree in *Cucumaria*. *Quart. J. Micr. Sci.*, **105**: 7–11.

FONTAINE, A. R., 1962. Neurosecretion in the ophiuroid, *Ophiopholes*. *Science*, **138**: 908–909.

LENTZ, T. H., AND R. J. BARRNETT, 1965. Fine structure of the nervous system of *Hydra*. *Amer. Zool.*, **5**: 341–356.

LEVER, J. D., J. D. P. GRAHAM, G. IRVINE AND W. J. CHICK, 1965. Vesicular axons in relation to arteriolar smooth muscle in the pancreas. (*Brit.*) *J. Anat.*, **99**: 299–313.

THAEMERT, J. C., 1966. Ultrastructural interrelationships of nerve processes and smooth muscle cells in three dimensions. *J. Cell Biol.*, **28**: 37–49.

THE DIGESTIVE SYSTEM OF THE HOLOTHURIAN, CUCUMARIA ELONGATA. I. STRUCTURE OF THE GUT AND HEMAL SYSTEM

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The structure and function of the echinoderm digestive system have been the subjects of several recent papers. Anderson (1953, 1959) has made valuable contributions to the study of digestion in asteroids, and both Stott (1955) and Fuji (1961) have studied the structural and functional aspects of the echinoid gut by means of histological and histochemical techniques. As early as 1883 Hamann gave detailed accounts of the gut histology of the holothurians, *Leptosynapta* and *Holothuria*, and more recently Stott (1957) has studied the alimentary canal and associated structures in *Holothuria forskali*. Choe (1962) has given an account of gut structure and digestive enzymes found in *Stichopus japonicus,* and the feeding and digestive processes of this holothurian have been studied by Tanaka (1958). However, the process of digestion in holothurians is still not fully understood. The function of the hemal system is open to controversy, and the role of the amoebocytes in digestion has yet to be conclusively demonstrated. To provide a fuller understanding of the process of digestion it is necessary for further detailed histological and histochemical studies to be accompanied by the results of physiological studies. This paper forms an introduction to the study of digestion in *Cucumaria elongata,* and deals with the histology and histochemistry of the gut. It is intended that a second paper will deal with the distribution of the digestive enzymes.

It is a pleasure to acknowledge the help and encouragement given by Dr. J. B. Buchanan, who supervised this study. Dr. D. B. Lewis gave valuable assistance with the photography. The work was supported by a research studentship from the Department of Scientific and Industrial Research.

Material and Methods

Specimens of *Cucumaria* were collected off the Northumberland coast from depths of about 20 fathoms. Those animals required for histological and histochemical studies were treated with a suitable fixative on the day of capture.

The different gut regions were dissected out in sea water and fixed in a suitable fluid. The material was processed and embedded according to the nature of the histological and histochemical techniques to be applied. (1) For general cell structure, tissues were fixed in Heidenhain's "Susa" made with sea water, embedded in paraffin wax and sectioned at 6 μ. For finer histological structure and the identification of secretory granules, tissues were fixed in Zenker-formol. (2) For the demonstration of mucin and similar compounds (acid polysaccharides), tissues fixed in Heidenhain's "Susa" were stained in dilute aqueous solutions of aluminum-methylene blue (Heath, 1962), alcian blue at pH 3, and mucicarmine. To demonstrate the metachromatic staining of acid polysaccharide elements, sections were stained overnight in dilute aqueous solutions of toluidine blue (0.01%). The pH at which the acid mucopolysaccharide lost the ability to bind with methylene blue (methylene blue extinction, M. B. E.) was determined by staining sections overnight in dilute solutions (0.01%) of aqueous methylene blue at different pH values. In all cases staining was followed by rapid dehydration in 95% and absolute alcohol. (3) For general recognition of lipid deposits tissues were fixed in Baker's formol-calcium, soaked in 5% potassium dichromate for 24 hours at 60° C., embedded in gelatine, sectioned at 10–15 μ on a freezing microtome, and stained with Sudan black. For the detection of phospholipid, material was fixed in Baker's formol-calcium and treated by Baker's acid-hematin method accompanied by the pyridine extraction test applied to sections fixed in weak Bouin's fluid (Baker, 1946). Material fixed in Baker's formol-calcium, post chromed, embedded in gelatine and sectioned as above, was stained in 1% aqueous Nile blue at 60° C. and differentiated in 1% acetic acid for the demonstration of acidic lipids (Cain, 1947). (4) For the demonstration of glycogen and related compounds, material was fixed in a weak Bouin's fluid, paraffin-embedded, and sections exposed to the periodic acid-Schiff reaction. Control slides exposed to the action of 1% malt diastase in a phosphate buffer at neutrality differentiate between glycogen and other Schiff-positive substances. (5) Identification of proteins. A full account of methods for the identification of proteins is given by Pearse (1960) in Appendix 5, page 791.

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(i) Identification of protein. Mercury-bromphenol blue method. (Formalin-fixed, paraffin-embedded.)
(ii) Identification of tyrosine. Millon reaction. (Baker modification).
(iii) Protein-bound NH_2. Ninhydrin-Schiff method. (Fixative: 85% ethanol. Paraffin sections.)
(iv) Identification of tryptophan.
  (a) Dimethylaminobenzaldehyde (D. M. A. B.) nitrate method. (Formalin-fixed, paraffin sections.)
  (b) Naphthyl ethylenediamine method: (Formalin-fixed, paraffin sections.) A stronger color was produced by this method than by the D.M.A.B. method.
(v) Identification of arginine. Sakaguchi reaction. (Susa-fixed, paraffin sections.)

GUT NOMENCLATURE AND MORPHOLOGY

There is confusion between the present systems of nomenclature used for the holothurian gut, primarily because of the morphological variation between species, and because the names of the different gut regions appear to have been assigned by analogy with the mammalian gut, rather than being based on functional differentiation. Stott (1957) has listed the nomenclatures used by Oomen (1926), Cuénot (1948) and Stott (1957). Choe (1962) has given a nomenclature for the gut of *Stichopus japonicus*, yet none of these is suitable for the gut of *Cucumaria*.

The system of nomenclature used throughout this study is as follows: pharynx, esophagus, stomach, constriction, intestine I, intestine II and cloaca. Each of these regions is morphologically clearly differentiated from the others (Fig. 1). By using terms which are familiar in the description of mammalian digestive systems, it is not intended that any functional comparisons should be drawn. Such names are retained only until a nomenclature based on functional differentiation can be given.

The first region of the gut, the *pharynx,* lies in the center of the aquapharyngeal bulb, and upon emergence into the body cavity it takes the form of the *esophagus.* The esophagus is slender yet conspicuous, having patches of black pigmentation at its anterior end. It is followed by a much broader and thicker-walled *stomach,* which is usually of similar length, but its pink coloration contrasts with the grey color of the esophagus. Following the stomach is a short, thin-walled region,

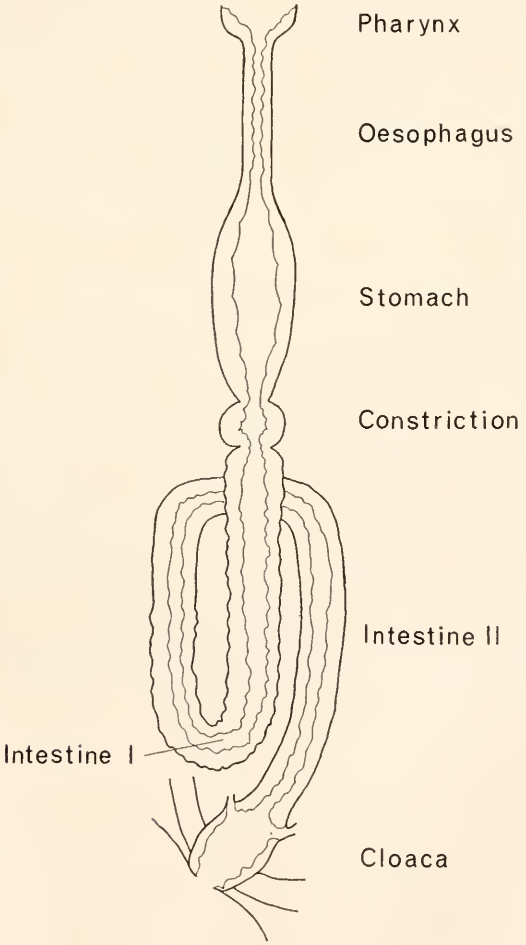
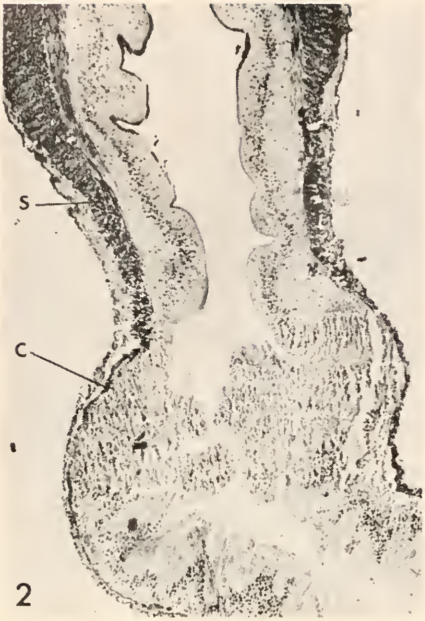
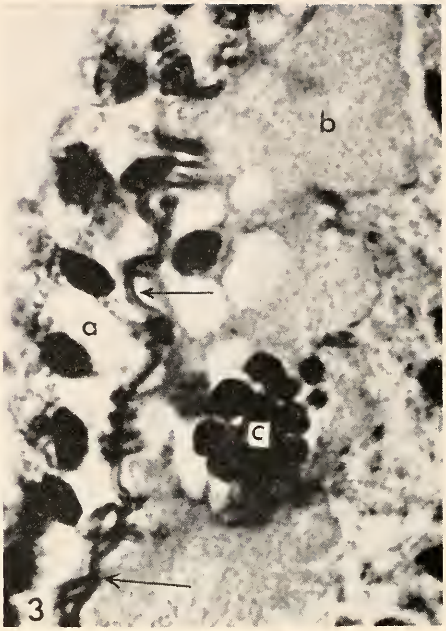


FIGURE 1. The gut of *Cucumaria elongata.* For details of gut nomenclature see text.





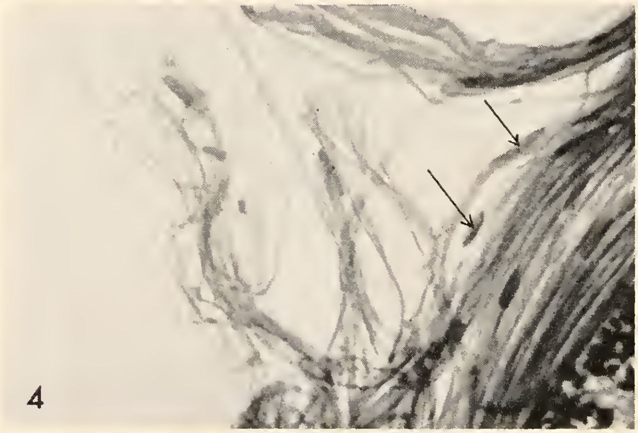


Figure 2. Longitudinal section through the junction between stomach and constriction. Compare the development of circular muscle in the stomach (S) with that in the constriction (C). Masson's trichrome. 1 cm. = 100 μ.

Figure 3. Transverse section of intestine I, showing muscle cell bodies (a), muscle layer (indicated by arrows), and connective tissue-fluid complex (b). C is an amoebocyte held in the fluid complex. Masson's trichrome. 1 cm. = 10 μ.

Figure 4. Circular muscle fibers of the stomach teased to show muscle cell bodies (indicated by arrows). Masson's trichrome. 1 cm. = 20 μ.

approximately 2–3 mm. long, which is bounded anteriorly and posteriorly by pronounced constrictions. Throughout this study this region of the gut is known as the *constriction.* The green color of the constriction contrasts vividly with the brown of the intestine and the pink of the stomach. The intestine of *Cucumaria* is typical of holothurians in being very long. *Intestine I* is about half as long again as *intestine II.* There is no color difference between these two parts, but the change from I to II is made clear by the change from the convoluted gut wall of intestine I to the relatively smooth-walled intestine II. The right and left respiratory trees open into the most posterior part of intestine II, from which point the gut is known as the *cloaca.* The *cloaca* is relatively short and runs to the posterior extremity of the animal.

Structure of the Gut

The gut wall consists of a number of distinct layers which can be described as follows:

(a) An outer covering of ciliated serosal epithelium which in places is so thin that it can only be detected by the presence of its nuclei.

(b) To the inside of the ciliated epithelium is a distinct layer of cells which are thought to be the cell bodies of the circular muscle fibers which lie to the inside of them.

(c) A muscle layer which is variously represented in the different regions of the gut. Outer circular and inner longitudinal muscle fibers are present in all gut regions. In the stomach the muscle bands show their maximum development, and are chiefly responsible for the thickness of the gut wall. At the junction between the stomach and the constriction there is a marked change in the musculature of the gut (Fig. 2). In the constriction the circular muscle is reduced to a band of fibers about 4 μ wide, while the longitudinal muscle is present as a few scattered fibers. This condition persists throughout the intestine and cloaca.

(d) A connective tissue layer, associated with which is a fluid continuous with that in the hemal system. The fluid component is variously represented in the different regions of the gut and is described fully below.

(e) The mucosal epithelium which, except in the stomach, is chiefly responsible for the thickness of the gut wall. It is composed of a single layer of tall slender cells, among which are several cell types described fully below.

Muscle cell bodies

A distinct layer of cells, varying from 10 to 15 μ thick and lying to the outside of the circular muscle (Fig. 3), is thought to comprise the cell bodies of the circular muscle fibers. The layer is present in all gut regions, and is covered by the serosal epithelium. In stained preparations cut both transversely and longitudinally it is difficult to interpret the relationship between the cell bodies and the fibers since both are densely packed. Even when pieces of the gut wall are teased and then stained with Masson's trichrome, the relationship is still obscure. There is no indication that these areas might be fiber bundles of a nerve layer, and the original contention that these cells are muscle cell bodies is held in view of the following observations. Preparations of esophagus, constriction and intestine, cut transversely and stained in Masson's trichrome, failed to show muscle cell bodies lying along the length of the circular fibers. In all the above regions the circular muscle fibers form a narrow layer, approximately 12–15 μ wide in the esophagus, compared with the thickness of a few fibers in the other regions. In these regions the muscle cell bodies must lie to the outside of the fibers. The cell bodies are highly distended and must be connected to individual fibers by way of short necks. In the stomach, where the circular muscle attains a thickness of 75 μ, muscle cell bodies can be clearly seen lying along the length of many of the fibers (Fig. 4). If the innermost fibers of the stomach were to have cell bodies arranged in the manner described for other gut regions, the connection between cell body and fiber would be *via* a neck in the region of 70 μ long. The presence of a thick overlying layer of densely packed muscle fibers makes such a connection unlikely. It is suggested that only the outermost circular fibers of the stomach have this highly distended type of cell body, whereas all fibers have this arrangement in the regions where the development of circular muscle is not as extensive. Although it has proved impossible to demonstrate conclusively that this arrangement exists, the proposed relationship between cell bodies and the individual muscle fibers is shown diagrammatically in Figure 5. Electron micrographs are necessary to give a clear picture of the arrangement which exists in this part of the gut wall.

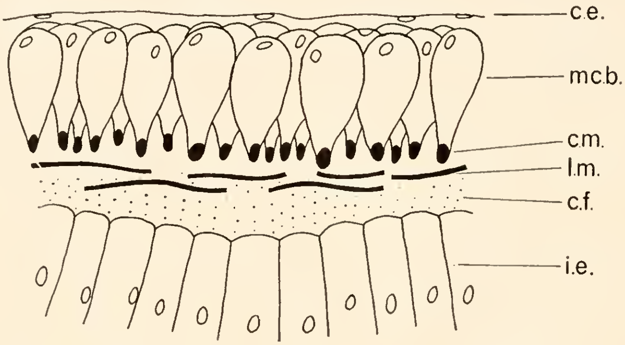


FIGURE 5. Semi-diagrammatic representation of the relationship between muscle cell bodies and the circular muscle fibers as seen in longitudinal section: c.e., coelomic epithelium; m.c.b., muscle cell bodies; c.m., circular muscle; l.m., longitudinal muscle; c.f., connective tissue-fluid complex; i.e., mucosal epithelium.

The connective tissue-fluid complex

The connective tissue layer attains its maximum development at the bases of the villus-like projections of the esophagus where it doubtless acts as a supporting framework. The most interesting aspect of this complex is the fluid component which is continuous with the fluid of the hemal system (Fig. 6). In living preparations the hemal fluid has a viscous appearance, whilst on fixation it appears to become "gelled." Amoebocytes are present in this fluid medium (see Fig. 3). Histochemical tests indicate that the fluid is periodic acid-Schiff-positive (diastase-fast) (Fig. 7), and contains tryptophan, arginine, tyrosine and reactive NH_2 groups, together with an acid mucopolysaccharide.

The mucosal epithelium

The mucosal epithelium is composed of a single layer of tall, slender cells which have centrally placed nuclei. Cells specialized to produce currents in the lumen of the gut are absent. In the esophagus the cells are formed into villus-like projections which have a connective tissue framework, and the individual cells are interspersed with large, conspicuous mucous gland cells (Fig. 8). Histochemical tests show that the glands contain an acid mucopolysaccharide with methylene blue extinction below pH 2. Negative results were obtained with the periodic acid-Schiff technique (Table I). Only a few of the glands extend to the basement membrane of the epithelium; those which do have a swollen basal portion (6–7 μ). The majority of the mucous glands are interspersed among the distal parts of the epithelial cells, and open by way of short necks into the esophageal lumen. In fixed preparations stained in aluminum-methylene blue, the contents of the glands appear distinctly granular. In the pharynx the mucous glands are similarly distributed.

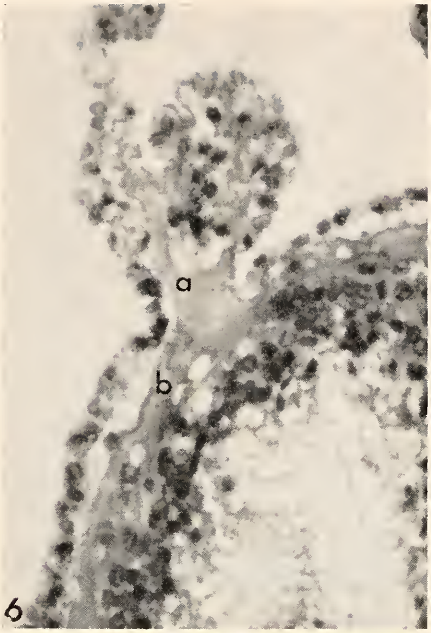
TABLE I

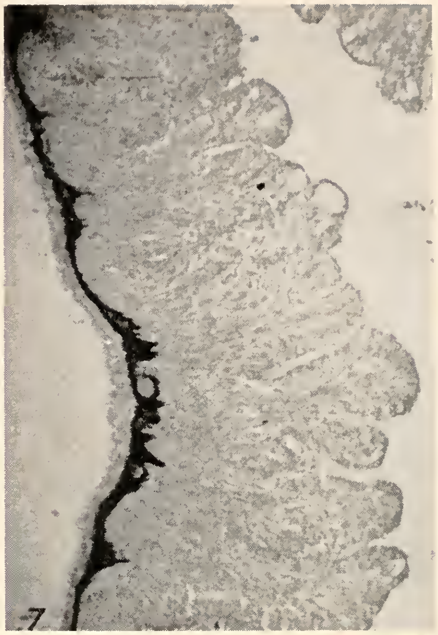
Histochemistry of the gland cells

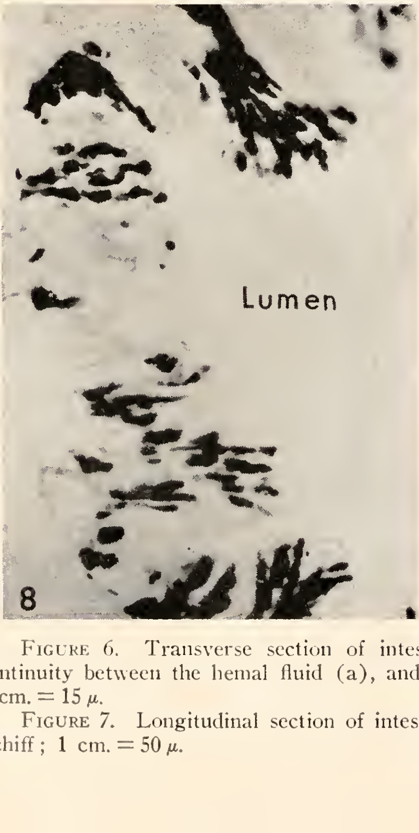
Test	Esophageal glands	Constriction glands	Intestine I glands
Toluidine blue	Gamma metachromasia	Gamma metachromasia	Negative
Alcian blue, pH 3	Positive	Positive	Negative
Aluminum-methylene blue	Positive	Positive	Negative
Mucicarmine	Positive	Positive	Negative
Methylene blue extinction	Below pH 2	Below pH 2	Negative to methylene blue
Periodic acid-Schiff reagent	Negative	Negative	Negative

In the stomach the cells of the mucosal epithelium are covered by a cuticle which has a thickness of about 2 μ (see Fig. 15). Mucous glands are absent and the gland cells which have been demonstrated by Hamann (1883) in the stomachs of *Leptosynapta* and *Holothuria* are lacking in *Cucumaria*. Chains of secretory granules found in the lining epithelial cells of the stomach of *Echinus esculentus* (Stott, 1955) and *Strongylocentrotus intermedius* (Fuji, 1961) have not been demonstrated, and the most conspicuous feature of the structure of the stomach wall of *Cucumaria* is its heavy musculature.

The epithelial cells of the constriction are formed into stout villus-like projections similar to those of the esophagus, and interspersed among individual cells there are numerous mucous gland cells which invariably extend to the basement membrane of the epithelium (Fig. 9). The base of the mucous glands has a diameter (6 μ) much greater than that of the neighboring epithelial cells. These glands show histochemical reactions similar to those of the esophagus (Table I), and when stained in aluminum-methylene blue the contents appear granular. Sections of the esophagus and constriction stained in aluminum-methylene blue, made with polychrome methylene blue (Microme salt no. 1041—E. Gurr), show differences in the staining reaction of the mucous glands after the preparations have been







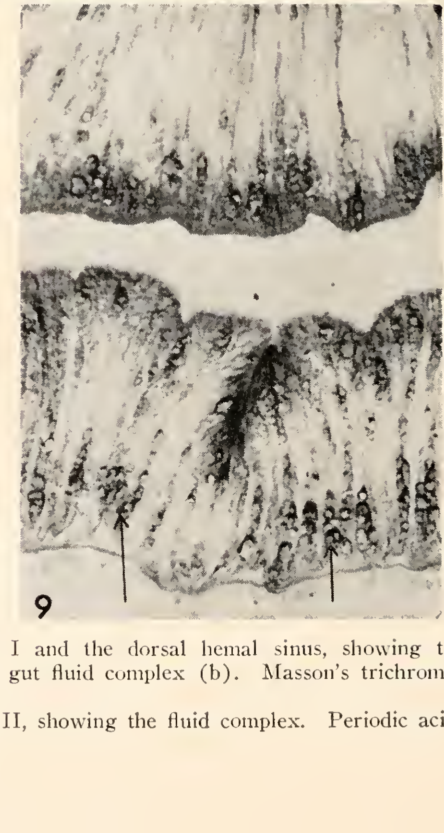


FIGURE 6. Transverse section of intestine I and the dorsal hemal sinus, showing the continuity between the hemal fluid (a), and the gut fluid complex (b). Masson's trichrome; 1 cm. = 15 μ.

FIGURE 7. Longitudinal section of intestine II, showing the fluid complex. Periodic acid-Schiff; 1 cm. = 50 μ.

stored for a few days. Mucous glands in the esophagus retain the brilliant blue-coloration characteristic of sulfated mucopolysaccharides, while those of the constriction change from blue to reddish purple. This would seem to indicate differences in the chemical nature of the secretion from these glands.

Preparations of the constriction stained in Heidenhain's iron hematoxylin and Masson's trichrome show two characteristic features. First, the swollen basal portion of the mucous glands is represented by light-colored areas in arcades between the bases of the epithelial cells. Secondly, there is a faintly stained "fringe" area, permeated by the ducts of the mucous glands, which represents the distal portion of the epithelial cells (Fig. 10). Throughout this fringe region histochemical tests show the presence of an acid mucopolysaccharide which has the same histochemical reactions as the glands of the esophagus and constriction, yet distinct gland cells are absent. Preparations stained in Heidenhain's iron hematoxylin also show secretory cells which contain chains of secretory granules (Fig. 11) similar to those described by Anderson (1953) in the pyloric caeca of *Asterias forbesi.* It has proved difficult to clearly establish the relationship between the secretory granules and the secretory cell, but in most cases the granules extend in rows towards the free end of the cell.

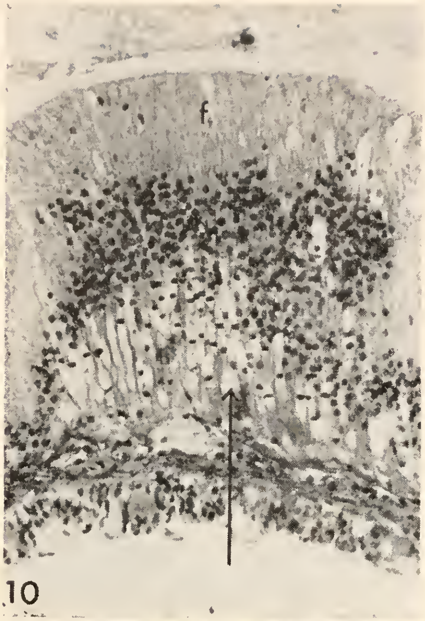
The organization of the epithelial cells throughout the intestine and cloaca is similar to that in the constriction, yet mucous glands and secretory granules are absent. Interspersed among the distal portions of the epithelial cells in intestine I, distinct gland cells are present which open into the lumen of the intestine (Fig. 12). These cells have only been demonstrated using Heidenhain's iron hematoxylin, and the contents appear granular. The nucleus is situated in the proximal half of the cell. Similar gland cells have been shown in the intestine of *Holothuria* by Hamann (1883). The distal portion of the epithelial cells of the intestine, corresponding to the "fringe" zone of the constriction, show faintly positive reactions for acid mucopolysaccharide. As in the "fringe" of the constriction, distinct gland cells are absent.

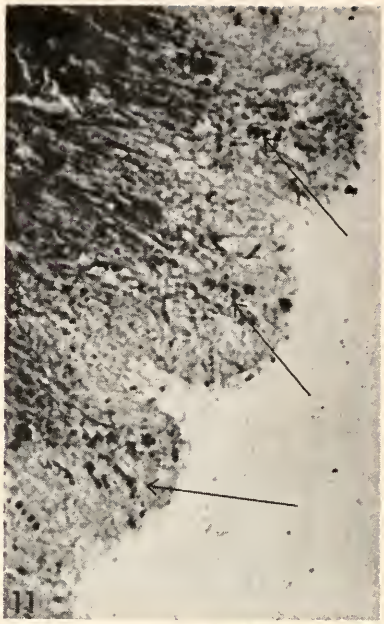
Storage cells

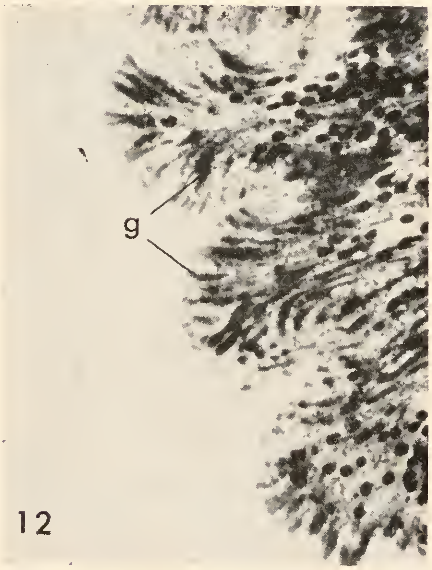
The mucosal epithelial cells in all regions of the gut hold deposits of lipid (Fig. 13), which constitutes an important food reserve of the animal (Fish, 1967). The lipid is stored in the form of droplets which lie both above and below the nucleus. In the distal region of the epithelial cells the droplets are generally small and sparsely distributed while in the basal portion they appear to have coalesced into larger globules. The Nile blue technique reveals that acidic lipids are prominent in the composition of the lipid deposits. The acid hematin test (Baker, 1946), accompanied by pyridine extraction, gave doubtful results for the presence of phospholipid. Lipid deposits are also present in the much inflated cell bodies of the circular muscular fibers. Sudan black staining shows that the muscle cell bodies are crowded with lipid droplets, the histochemistry of which is the same as that of lipid stored in the epithelial cells.

FIGURE 8. Transverse section of esophagus, showing mucous glands. Aluminum-methylene blue; 1 cm. = 35 μ.

FIGURE 9. Longitudinal section of the constriction, showing mucous glands (indicated by arrows). Aluminum-methylene blue; 1 cm. = 35 μ.







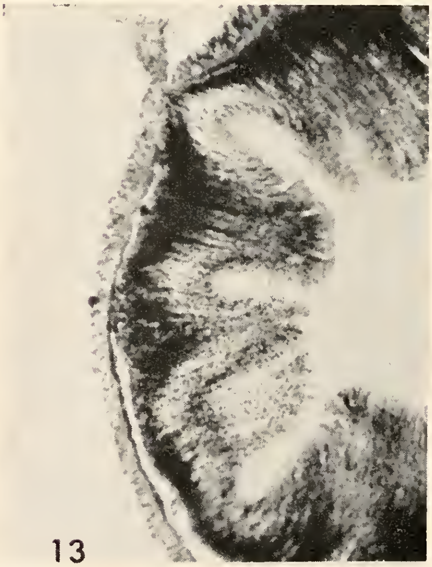


FIGURE 10. Transverse section of the constriction. Basal portion of mucous glands indicated by arrow. Note lightly stained "fringe" zone at f. Masson's trichrome; 1 cm. = 40 μ.

FIGURE 11. Transverse section of the constriction, showing chains of secretory granules. Heidenhain's iron hematoxylin; 1 cm. = 30 μ.

FIGURE 12. Longitudinal section of intestine I, showing gland cells (g). Heidenhain's iron hematoxylin; 1 cm. = 30 μ.

FIGURE 13. Transverse section of the constriction, showing lipid deposits. Note concentration of lipid in basal portion of cells. Frozen sections, Sudan black; 1 cm. = 55 μ.

THE HEMAL SYSTEM

Typical of holothurians there is a close association between the gut and the hemal system. The system in *Cucumaria* is shown diagrammatically in Figure 14, and consists of two main sinuses—the dorsal and the ventral. There is no rete mirabile or complicated network of lacunar tufts such as is found in *Holothuria forskali* (Stott, 1957) and other large aspidochirotes, yet transverse connections between different parts of the same sinus are evident (Fig. 14). A direct route between the hemal system and the gut is provided by the continuity which exists

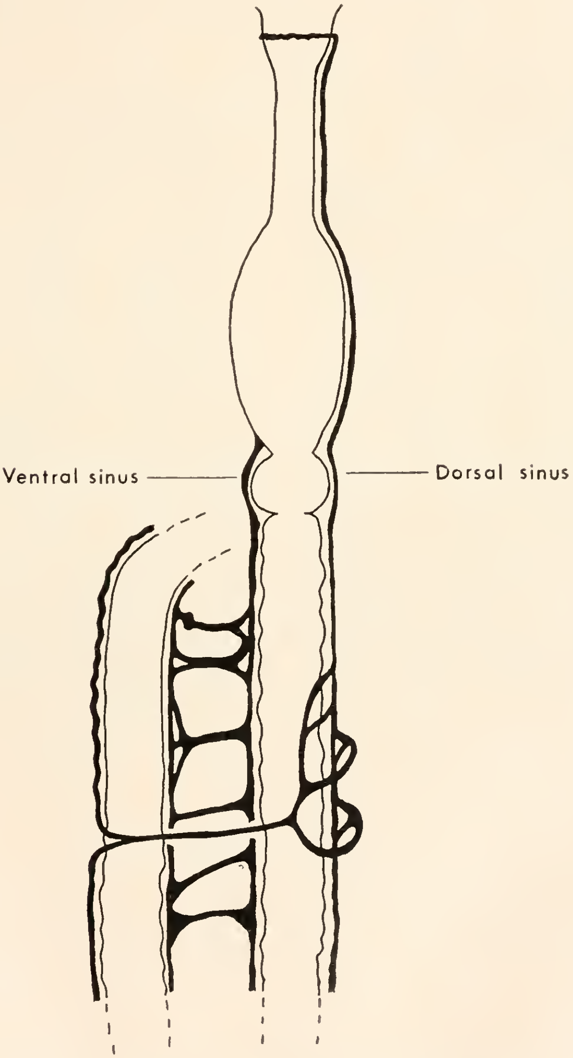


FIGURE 14. Hemal system of *Cucumaria*, showing dorsal and ventral sinuses and their connecting strands.

between the hemal fluid and the connective tissue-fluid complex of the gut (see Fig. 6).

The ventral sinus runs along the length of the intestine and constriction, yet at the anterior end of the constriction the sinus ceases to exist as a separate channel, and serial sections at this point show that it passes diffusely through the stomach wall until it reaches the connective tissue-fluid complex (Fig. 15). The author is not aware of a similar system in any other holothurian; the more usual arrangement is for the ventral vessel to continue along the length of the stomach and esophagus until it reaches the hemal ring surrounding the pharynx.

Throughout its course the ventral sinus is in close association with the gut wall. At the anterior region of intestine I the sinus gives off several transverse connections which join with that part of the ventral sinus which is associated with the posterior part of intestine I and the anterior part of intestine II.

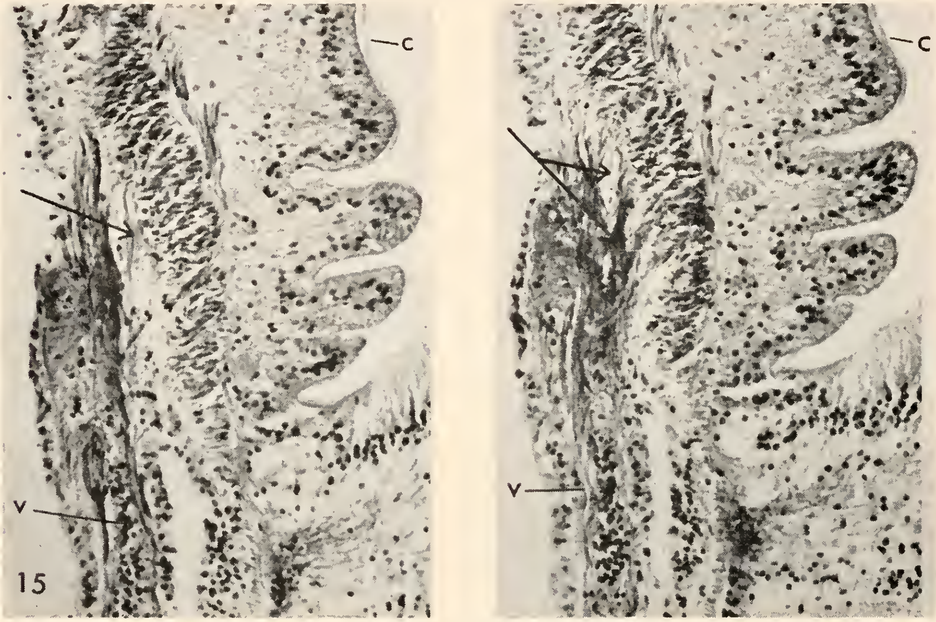
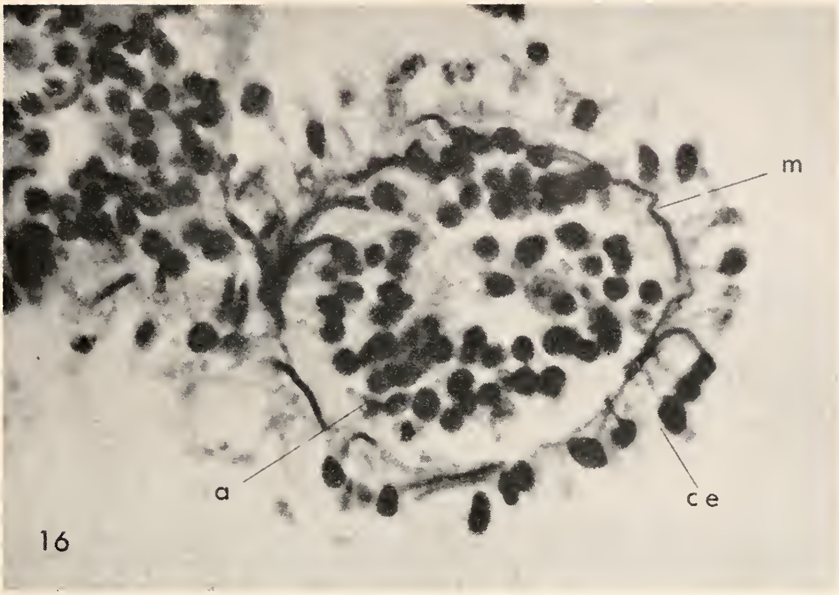


FIGURE 15. Serial longitudinal sections through the junction between stomach and constriction, showing the ventral hemal sinus (v) merging with the stomach wall at points indicated by arrows. Note the cuticle (c) covering the mucosal epithelial cells of the stomach. Masson's trichrome; 1 cm. = 40 μ.

The dorsal sinus runs along the complete length of the gut on the side which is attached by the dorsal mesentery. It is connected to the intestinal wall by numerous branches, and shortly after the commencement of its course along intestine I, it gives off a single transverse connection which joins the part of the dorsal sinus which is associated with the anterior part of intestine II. Anteriorly the dorsal sinus diminishes towards the pharynx. The presence of a hemal ring has not been satisfactorily demonstrated, yet this may be due to its delicate nature and the fact that it is believed to lie directly behind the water vascular ring.



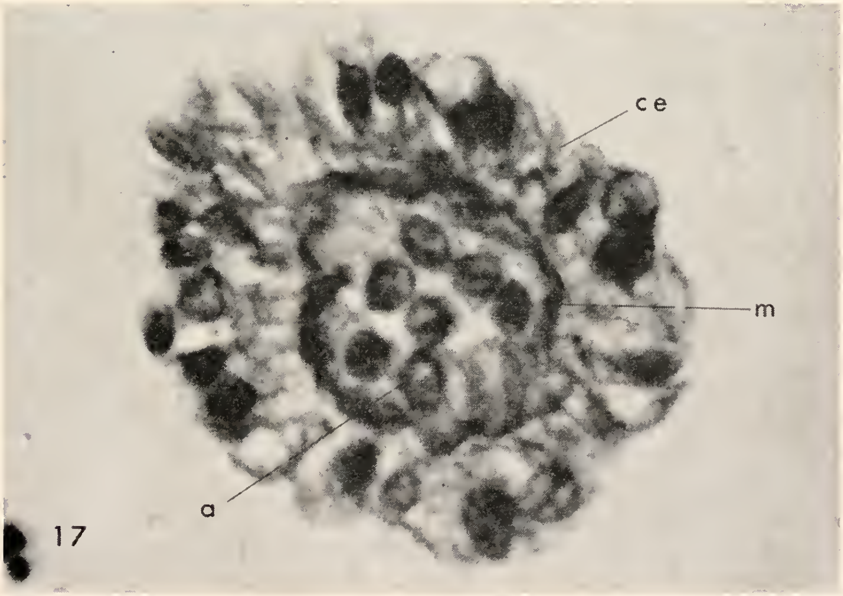


FIGURE 16. Dorsal hemal sinus seen in transverse section. Note coelomic epithelium, ce; muscle layer, m; and amoebocytes within the sinus (a). Heidenhain's iron hematoxylin, 1 cm. = 10 μ.

FIGURE 17. Transverse connecting strand of the ventral hemal sinus seen in transverse section. Note coelomic epithelium, ce; muscle layer, m; and amoebocytes within the sinus (a), Masson's trichrome; 1 cm. = 10 μ.

Although the small size and delicate nature of the hemal sinuses make it difficult to obtain good sections, available evidence suggests that all parts of the system have the same histological structure (Figs. 16 and 17). There is an outer layer of coelomic epithelium which contains strands of connective tissue. The thickness of this layer varies from 4–5 μ to 10–12 μ. A thin but distinct layer of circular muscle fibers is found to the inside of the coelomic epithelium. Associated with the circular muscle fibers there are a few scattered longitudinal fibers, to the inside of which is an indistinct layer of connective tissue. In all sections of the hemal sinuses the circular muscle fibers have shown as a distinct layer. This contrasts with *Stichopus chloronotus* (Sivickis and Domantay, 1928), which has an indistinct muscle layer in the hemal sinus. The lacunar tufts of the rete mirabile of *Actinopyga* were found by Hyman (1955) to be without a muscle layer.

Sections of the hemal sinuses treated with aluminum methylene blue and the periodic acid-Schiff technique gave negative results.

Discussion

The results of histological and histochemical observations on the gut of *C. elongata* present several interesting features. The relationship between the muscle cell bodies and the individual circular muscle fibers poses problems as to the reasons for, or advantages of, such a system. Nichols (1959) has described a similar arrangement of muscle cell bodies in the ampullae of the tube feet of *Echinocardium.* Such an arrangement has not previously been recorded in the gut wall of holothurians. The development of this system is perhaps associated with the lack of connective tissue in the outer layers of the gut wall. The layer of inflated, densely packed and interlocking muscle cell bodies may function as an anchorage system for the circular muscle fibers. In the absence of connective tissue in this region, such an arrangement would be important during phases of strong muscle contraction.

Lipid stores are held in the muscle cell bodies as well as in the cells of the mucosal epithelium. Nichols (1959) found that in *Echinocardium,* glycogen was held in the muscle cell cytoplasm as a food store. Glycogen has not been detected in either the body wall or gut of *Cucumaria.* The failure to obtain a positive Baker test for the presence of phospholipid is interesting, in that Anderson (1953) and Karnovsky *et al.* (1955) failed to demonstrate phospholipid in *Asterias.* However, as pointed out by Karnovsky *et al.,* the failure of the test may be due to the low phosphorus content of the phosphatide fraction and not due to the low concentration of phosphatide. In the acid hematin test, the hematin is presumed to react with the phosphate radical. Although the specificity of the Baker test is established (Casselman, 1952), its sensitivity has never been determined, and it may be that in spite of negative results, phospholipids are present in the constitution of the lipid deposits. It must also be emphasized that as marine invertebrates have low melting point lipids (Giese, 1966), the technique used in the histochemical localization of the lipid deposits is itself questionable, as it involved incubation in 5% potassium dichromate for 24 hours at 60° C.

Results similar to those obtained for the histochemistry of the hemal fluid in *Cucumaria* have been recorded by Millot and Vevers (1964) for the axial organ secretion in echinoids. These authors suggest that the axial organ is suitably positioned to act as an endocrine organ, and they have shown that considerable quantities of secretion leave the glandular recesses of the organ. Millot (1966) has further suggested that the reactions of the axial organ may be part of a "defensive injury response." In holothurians there is little agreement between authors on the existence of an axial gland. Cuénot (1891) claims that the part of the coelom giving rise to the axial gland disappears during embryonic development, while other workers described a connective tissue network to the side of the water ring which they considered to be an axial gland (*vide* Hyman, 1955). It is significant to note that even though the axial organ in echinoids may secrete fluid into the hemal system, Millot and Vevers (1964) believed it unlikely that amoebocytes arose there. Furthermore, Holland *et al.* (1965) were unable to find evidence either for or against the participation of the axial organ in amoebocyte production.

Although the structure and possible functions of the hemal system have been investigated by a number of authors, the functions of the system have not been conclusively demonstrated. A number of investigators have reported a contractile nature for parts of the hemal system (Kawamoto, 1927; Prosser and Judson, 1952; Boolootian and Campbell, 1964; see also Hyman, 1955). Prosser and Judson (1952) further demonstrated that in holothurians the contractions were myogenic, being accelerated by adrenalin and slowed by atropin. Burton (1964) has shown that despite evidence of contractility of the sinus in regular echinoids, the full significance of this is not yet clear, and it would appear unlikely that the hemal system functions as a true circulatory system. The experiments and histological observations of Enriques (1902), Oomen (1926), and Schreiber (1930, 1932a, 1932b), led to the hypothesis that the holothurian hemal system played an important role in digestion, in that amoebocytes contained within the hemal fluid were believed to carry digestive enzymes into the gut, and carry away the products of digestion. Contrary to these earlier reports it has recently been suggested that sugars may cross the gut wall by active transport (D'Agostino and Farmanfarmaian, 1960; Rundles and Farmanfarmaian, 1964). It has further been shown that the hemal sinuses are not significantly involved in nutrient transport in either echinoids or holothurians (Farmanfarmaian and Phillips, 1962; Farmanfarmaian, 1963). Results of histochemical tests applied to sections of gut material of *Cucumaria* are also contrary to the hypothesis of Enriques, Oomen and Schreiber. These results show that parts of the mucosal epithelium of the gut appear to be capable of secreting digestive enzymes. The constriction has abundant mucous glands and chains of secretory granules, and intestine I has conspicuous gland cells. The distribution of mucous glands, secretory granules and gland cells would appear to indicate that at least the constriction and intestine I are sites of enzyme production and secretion. The possibility of ascribing a zymogenic function to parts of the lining epithelium of the gut will be considered more closely in the second part of this study when the results of the distribution of digestive enzymes are discussed.

SUMMARY

1. A system of nomenclature is given for the gut of *Cucumaria elongata*. The different regions of the gut have been named as follows: pharynx, esophagus, stomach, constriction, intestine I, intestine II, and cloaca.

2. The gut wall is composed of five distinct layers: (a) an outer serosal epithelium; (b) muscle cell bodies of the circular muscle fibers; (c) a muscle layer with outer circular and inner longitudinal fibers; (d) a connective tissue-fluid complex, the fluid component of which is continuous with the fluid in the hemal system; (e) the mucosal epithelium, which is composed of a single layer of tall slender cells.

3. Interspersed among the cells of the mucosal epithelium are mucous glands, secretory granules and gland cells. Mucous glands are present in the esophagus and constriction; secretory granules in the constriction, and gland cells in intestine I. Cells specialized to produce currents in the lumen of the gut are absent.

4. Stores of lipid are held in the cells of the mucosal epithelium and in the muscle cell bodies of the circular muscle fibers. Glycogen deposits have not been demonstrated.

5. The histology of the hemal system has been studied and the role of the hemal system in digestion is discussed.

6. From the distribution of gland cells and secretory granules it is suggested that the mucosal epithelial cells of the constriction and intestine I are sites of digestive enzyme production and secretion.

LITERATURE CITED

ANDERSON, J. M., 1953. Structure and function in the pyloric caeca of *Asterias forbesi*. *Biol. Bull.*, **105**: 47–61.

ANDERSON, J. M., 1959. Studies on the cardiac stomach of a starfish, *Patiria miniata* (Brandt). *Biol. Bull.*, **117**: 185–201.

BAKER, J. R., 1946. The histochemical recognition of lipine. *Quart. J. Micr. Sci.*, **87**: 441–447.

BOOLOOTIAN, R. A., AND J. L. CAMPBELL, 1964. A primitive heart in the echinoid *Strongylocentrotus purpuratus*. *Science*, **145**: 173–175.

BURTON, M. P. M., 1964. Hemal system of regular echinoids. *Nature*, **204**: 1218.

CAIN, A. J., 1947. The use of Nile Blue in the examination of lipoids. *Quart. J. Micr. Sci.*, **88**: 383–392.

CASSELMAN, W. G. B., 1952. Observations concerning the specificity of the acid haematin test for phospholipids. *Quart. J. Micr. Sci.*, **93**: 381–383.

CHOE, S., 1962. Biology of the Japanese common sea cucumber, *Stichopus japonicus* Selenka. 226 pp. Pusan, Korea. (In Japanese with English summary.)

CUÉNOT, L., 1891. Études morphologiques sur les échinodermes. *Arch. Biol.*, **11**: 313–608.

CUÉNOT, L., 1948. Anatomie, ethologie et systematiques des échinodermes. *In:* Traité de Zoologie, ed. P. Grassé, T. XI. Masson, Paris.

D'AGOSTINO, A. S., AND A. FARMANFARMAIAN, 1960. Transport of nutrients in the holothurian *Leptosynapta inhaerens*. *Biol. Bull.*, **119**: 301.

ENRIQUES, P., 1902. Digestione, circolazione e assorbimento nelle oloturie. *Arch. Zool. Ital.*, **1**: 1–58.

FARMANFARMAIAN, A., 1963. Transport of nutrients in echinoderms. *Proc. XVI. Int. Cong. Zool.*, **1**: 118.

FARMANFARMAIAN, A., AND J. H. PHILLIPS, 1962. Digestion, storage and translocation of nutrients in the purple sea urchin *Strongylocentrotus purpuratus*. *Biol. Bull.*, **123**: 105–120.

FISH, J. D., 1967. The biology of *Cucumaria elongata*. (Echinodermata: Holothuroidea.) *J. Mar. Biol. Assoc.*, **47**: 129–143.

FUJI, A., 1961. Studies on the biology of the sea urchin, IV. Histological observation of the food canal of *Strongylocentrotus intermedius*. *Bull. Fac. Fisheries, Hokkaido Univ.*, **11**: 195–202.

GIESE, A. C., 1966. Lipids in the economy of marine invertebrates. *Physiol. Reviews,* **46**: 244–298.

GURR, E., 1960. Encyclopaedia of Microscopic Stains. Leonard Hill, London.

HAMANN, O., 1883. Beiträge zur Histologie der Echinodermen. Die Holothurien. *Zeitschr. wiss. Zool.*, **39**: 145–190.

HEATH, I. D., 1962. Observations on a highly specific method for the histochemical detection of sulphated mucopolysacharides, and its possible mechanisms. *Quart. J. Micr. Sci.*, **103**: 457–475.

HOLLAND, N. D., J. H. PHILLIPS AND A. C. GIESE, 1965. An autoradiographic investigation of coelomocyte production in the purple sea urchin (*Strongylocentrotus purpuratus*). *Biol. Bull.*, **128**: 259–270.

HYMAN, L. H., 1955. The Invertebrates: Echinodermata. The Coelomate Bilateria. McGraw-Hill Book Co., New York.

KAWAMOTO, N., 1927. Anatomy of *Caudina chilensis*. *Tôhoku Univ. Sci. Repts. Ser. 4 Biol.*, **2**: 239–265.

KARNOVSKY, M. L., S. S. JEFFRY, M. S. THOMPSON AND H. W. DEANE, 1955. A chemical and histochemical study of the lipids of the pyloric caecum of the starfish *Asterias forbesi*. *J. Biophysic. Biochem. Cytol.*, **1**: 173–182.

MILLOT, N., 1966. A possible function for the axial organ of echinoids. *Nature*, **209**: 594–596.

MILLOT, N., AND H. G. VEVERS, 1964. Axial organ and fluid circulation in echinoids. *Nature*, **204**: 1216–1217.

NICHOLS, D., 1959. The histology of the tube feet and clavulae of *Echinocardium cordatum*. *Quart. J. Micr. Sci.*, **100**: 73–87.

OOMEN, H. A. P. C., 1926. Verdauungsphysiologische Studien an Holothurien. *Pubbl. Staz. Zool. Napoli*, **7**: 215–297.

PEARSE, A. G. E., 1960. Histochemistry. Theoretical and Applied. Churchill, London.

PROSSER, C. L., AND C. L. JUDSON, 1952. Pharmacology of the haemal vessels of *Stichopus*. *Biol. Bull.*, **102**: 249–251.

RUNDLES, C., AND A. FARMANFARMAIAN, 1964. Absorption and transport of D-glucose in the intestine of *Thyone briareus*. *Biol. Bull.*, **127**: 387–388.

SCHREIBER, B., 1930. Studi sull' assorbimento intestinale nelle oloturie. *Pubbl. Staz. Zool. Napoli*, **10**: 235–277.

SCHREIBER, B., 1932a. Pigmenti e secrezioni nel sistema digerente nelle oloturie. *Pubbl. Staz. Zool. Napoli*, **12**: 18–60.

SCHREIBER, B., 1932b. Experimenti per lo studio dell' assorbimento intestinale nelle oloturie. *Arch. Zool. Ital.*, **16**: 865–870.

SIVICKIS, P., AND J. DOMANTAY, 1928. The morphology of a holothurian, *Stichopus chloronotus* Brandt. *Phillipine J. Sci.*, **37**: 229–332.

STOTT, F. C., 1955. The food canal of the sea urchin *Echinus esculentus L.* and its functions. *Proc. Zool. Soc. London*, **125**: 63–86.

STOTT, F. C., 1957. Observations on the food canal and associated structures in the holothurian *Holothuria forskali* Delle Chiaje. *Proc. Zool. Soc. London*, **129**: 129–136.

TANAKA, Y., 1958. Feeding and digestive processes of *Stichopus japonicus*. *Bull. Fac. Fisheries, Hokkaido Univ.*, **9**: 14–28.

THE DIGESTIVE SYSTEM OF THE HOLOTHURIAN, CUCUMARIA ELONGATA. II. DISTRIBUTION OF THE DIGESTIVE ENZYMES

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Several enzymes have been recorded from holothurian digestive tracts and in extracts of the gut wall. Oomen (1926) demonstrated a protease, amylase, maltase and a weak lipase in *Holothuria*, and Van der Heyde (1922) reported protease, invertase and lipase, but no amylase in *Thyone*. The digestive fluid and extracts of the gut wall of *Caudina chilensis* were found by Sawano (1928) to contain lipase, maltase, invertase, glycogenase and a protease resembling trypsin. Choe (1962) detected amylase, cellulase, pectinase and dipeptidase in gut extracts of both the green and red varieties of *Stichopus japonicus*. He also demonstrated small amounts of lipolytic enzyme capable of digesting simple ester, glyceride and higher fatty acid. It is now generally accepted that holothurians have abundant carbohydrate-splitting enzymes and a proteolytic enzyme similar to trypsin. Although Choe (1962) has given details of the amount of enzyme activity recorded from the different parts of the intestine of *Stichopus japonicus*, there is little information available concerning the distribution of digestive enzymes in holothurians. In the present study, extracts of the different gut regions of *Cucumaria* were tested for proteases, carbohydrases and lipases, so as to determine the distribution of enzymes as well as the enzyme complement.

It is interesting to note that Pequignat (1966) has recently demonstrated "skin digestion" in echinoids. He has shown that the mucous coating of the body surface is capable of digesting a wide range of food materials. He observed spherule coelomocytes "creeping" out to the external mucous coating where they eventually disintegrated "while probably releasing digestive enzymes." Although Pequignat has not studied holothurians, he claims that it is unlikely that they should behave any differently. However, in terms of overall nutritional requirements it is unlikely that "skin digestion" can be of serious significance to animals with well developed digestive tracts.

I am grateful to Dr. J. B. Buchanan for advice and encouragement. The work was supported by a research studentship from the Department of Scientific and Industrial Research.

MATERIALS AND METHODS

Specimens of *Cucumaria* were collected off the Northumberland coast during July and August, 1966, and the enzyme extract was prepared on the day of capture.

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Preparation of enzyme extract

Extracts were prepared of the esophagus, stomach, constriction, intestine I and intestine II. Details of gut nomenclature are given by Fish (1967).

The different regions of the gut were carefully dissected from a number of animals and placed in separate containers. After removal of the gut contents the material was quickly rinsed and then blotted dry. The gut material was weighed and added to twice its own weight of glycerol and homogenized for 10 minutes in an M.S.E. homogenizer. The volume obtained was diluted with an equal volume of filtered sea water and centrifuged for 15 minutes. The supernatant was collected and sea water of an equal volume to this supernatant was added *to the residue* which, after mixing, was centrifuged for a further 15 minutes. The second supernatant was added to the first to give the final enzyme extract which was filtered through a Whatman No. 4 paper. Toluene was added to prevent putrefaction. Incubation with substrate solutions was started on the day following preparation, the extracts being stored overnight at 4° C.

Estimation of proteases

Proteolytic enzymes were estimated using the formol titration method of Sørensen, as described by Davis and Smith (1955). For speed and convenience titration by indicator was preferred to the potentiometric titration recommended by Dunn and Loshakoff (1936). One-tenth per cent phenolphthalein in absolute alcohol was used as the indicator, and the enzyme-substrate mixture was titrated against approximately 0.3 N NaOH. For all titrations an "Alga" micrometer syringe was used instead of a burette. The volume of alkali delivered was accurate to 0.001 ml. The results are expressed as the amount of hydrolysis per hour per ml. of enzyme extract by using arbitrary units, *i.e.*, 0.01 ml. of 0.3 N NaOH = 10 units of hydrolysis.

The enzymes studied, together with the respective substrate solutions, were as follows:

Enzyme system	Substrate (1% aqueous solution)
Trypsin	α Benzoyl-L-arginine, ethyl ester
Aminotripeptidase	Triglycine
Glycylglycine dipeptidase	Glycylglycine
Leucine aminopeptidase	L-Leucyl-glycylglycine
Carboxypolypeptidase	Chloracetyl-L-tyrosine

Estimation of carbohydrases

Amylase, invertase and maltase were estimated by using starch, sucrose and maltose solutions as the respective substrates.

One ml. of gut extract plus 2 ml. of substrate solution were incubated for 12 hours at 20° C. Quantitative estimations were carried out by volumetric estimation of the cuprous oxide which was formed on reduction of a cupric salt by the products of enzyme hydrolysis. The method employed was that of Bertrand, described by Plimmer (1920) with the following modifications.

Instead of boiling the reagent-sugar mixture over a bunsen flame for three minutes the mixture was heated in a boiling water bath for 15 minutes. It was cooled and centrifuged at 3500 r.p.m. for 15 minutes, and the supernatant carefully decanted, leaving a deposit of cuprous oxide. Centrifugation is quicker and less tedious than removing the precipitate by filtration through a special asbestos filter as described in the original method. It was found necessary to avoid transference of the solution from one tube to another because the cuprous oxide becomes adsorbed onto the walls of the tube as it is being precipitated. Transferance of the solution was found to result in a considerable loss of cuprous oxide. Consequently, the whole procedure, from incubation to titration, was carried out in the same tube. As a control experiment 2 ml. of substrate solution were incubated without gut extract and treated as described above, so as to account for any hydrolysis of the substrate not due to enzyme action.

The modified Bertrand method is satisfactory when dealing with the quantitative estimation of amylase, and invertase, as the substrate solutions used are not reducing sugars. However, as the method depends upon the reduction of alkaline cupric sulfate by the products of enzyme hydrolysis—reducing sugars—it is useless when testing for maltase, as the substrate used, maltose, is itself a reducing sugar. To overcome this, 10 ml. of cupric acetate solution (Barfoed's reagent) were used in place of alkaline cupric sulfate. Glucose, the product of enzyme hydrolysis, reduces Barfoed's reagent, giving a precipitate of red cuprous oxide, whereas it is unaffected by maltose.

Titration values were converted to mg. of glucose liberated by enzyme hydrolysis by reference to calibration curves obtained with glucose under conditions stated above. Although invertase on hydrolysis yields molecules of fructose as well as molecules of glucose, the reducing power of fructose is so similar to that of glucose (Plimmer, 1920), that for the purpose of constructing the calibration curves, the products of enzyme hydrolysis were regarded as molecules of glucose. Results are expressed as mg. of glucose liberated per hour per ml. of enzyme extract.

Qualitative estimation of carbohydrases

The extract used in the qualitative estimation of carbohydrases was prepared by the technique described above, using the complete gut from several animals. In each test 1 ml. of extract was incubated at 20° C. for 12 hours with 2 ml. of the respective substrate solution. Tests were made for amylase, invertase, maltase, lactase, glycogenase and cellulase.

Supplies of Laminarin and Fucoidin, substrates for cellulase activity, were obtained in the soluble powder form from the Seaweed Research Institute, Inveresk, Midlothian, Scotland.

Estimation of esterase and lipase

Esterase and lipase were estimated quantitatively by using the method of Nachlas and Seligman (1949). Beta-naphthyl esters were used as substrates. Broadly speaking, the esters of short-chain fatty acids (C_2-C_4) are split by esterases, and the long-chain esters (C_8-upwards) by lipases, yet according to Nachlas and Seligman (1949), there is a considerable degree of overlapping in enzymatic hydrolysis by these two enzymes. Enzymes splitting the substrate beta-naphthyl acetate (C_2) are here regarded as esterases, those splitting beta-naphthyl

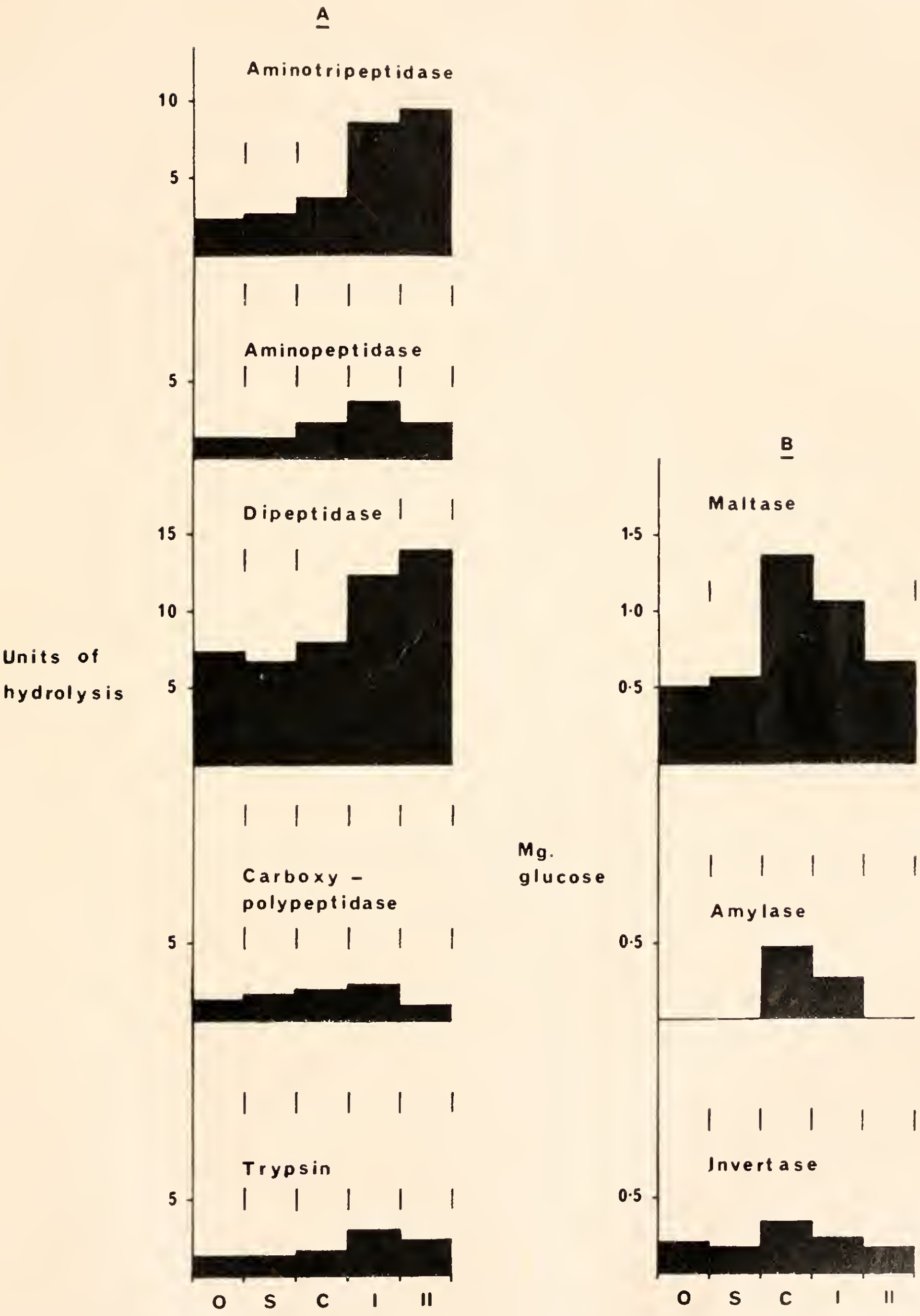

FIGURE 1. Enzyme activity recorded from the different regions of the gut. A, proteases; B, carbohydrases; O, esophagus; S, stomach; C, constriction; I, intestine I; II intestine II.

TABLE I

Activity of proteolytic enzymes from the different gut regions

	Amino-tripeptidase	Amino-peptidase	Dipeptidase	Carboxy-polypeptidase	Trypsin
Esophagus	2.2 ± 0.9	1.4 ± 0.4	7.4 ± 1.4	1.2 ± 0.5	1.3 ± 0.2
Stomach	2.6 ± 1.4	1.4 ± 1.0	6.6 ± 1.7	1.4 ± 0.4	1.2 ± 0.4
Constriction	3.7 ± 1.4	2.4 ± 1.1	8.0 ± 0.7	1.8 ± 0.8	1.6 ± 1.0
Intestine I	8.9 ± 2.1	3.8 ± 1.3	12.2 ± 2.1	2.0 ± 0.7	3.0 ± 1.3
Intestine II	9.8 ± 4.5	2.5 ± 0.3	14.0 ± 2.5	1.1 ± 0.6	2.2 ± 0.8

Activity expressed as units of hydrolysis/ml. of extract/hr. Each value is the mean and standard deviation of 5 determinations.

laurate (C_{12}) are described as "esterase-lipase" and enzymes splitting beta-naphthyl stearate (C_{18}) as lipases.

Beta-naphthol is liberated by enzymatic hydrolysis and an azo dye is produced by coupling the free naphthol with a tetrazonium salt. The colored compound was extracted with ethyl acetate and measured colorimetrically. All measurements were made using the Unicam Spectrophotometer S.P.600 at a wave-length of 540 mμ. The colorimeter readings were converted to mg. of beta-naphthol by reference to a calibration curve obtained using known quantities of beta-naphthol. The results are expressed as mg. beta-naphthol liberated per hour per ml. of enzyme extract.

RESULTS AND CONCLUSIONS

Results are featured in Tables I–IV, and Figure 1, and indicate the presence of a variety of digestive enzymes in gut extracts of *Cucumaria*. An endopeptidase of a trypsin-like nature and several exopeptidases are present, and although these were detected in extracts of all gut regions, maximum activity was without exception recorded from the intestine. Maltase, amylase, and invertase were readily detected, each having maximum activity in the constriction, and glycogenase has been detected in extracts of the whole gut. Lactase and cellulase have not been detected in *Cucumaria*. The distribution of amylase is interesting, in that of the enzymes

TABLE II

Results of the qualitative estimation of carbohydrases

Enzyme	Substrate	Reagent employed	Result
Amylase	1% Starch soln.	Fehling's soln.	++
Invertase	5% Sucrose soln.	Fehling's soln.	++
Maltase	5% Maltose soln.	Barfoed's reagent	++
Lactase	2% Lactose soln.	Barfoed's reagent	−
Glycogenase	Saturated soln. of glycogen	Fehling's soln.	+
Cellulase	1% Laminarin soln. 1% Fucoidin soln. 1% Sodium alginate	Fehling's soln.	−

++ = Strongly positive; + = positive; − = negative.

TABLE III

Activity of carbohydrases from the different gut regions

	Maltase	Invertase	Amylase
Esophagus	0.51 ± 0.2	0.20 ± 0.3	0
Stomach	0.58 ± 0.2	0.17 ± 0.2	0
Constriction	1.38 ± 0.3	0.34 ± 0.2	0.49 ± 0.2
Intestine I	1.07 ± 0.2	0.25 ± 0.3	0.26 ± 0.2
Intestine II	0.66 ± 0.2	0.16 ± 0.1	0

Activity expressed as mg. reducing sugar/ml. of extract/hour. Each value is the mean and standard deviation of 5 determinations.

studied, it is the only one which is not found throughout the gut. Choe (1962) detected amylase in the first and second small intestines and the anterior and posterior parts of the large intestine of *Stichopus japonicus.*

A strong esterase activity and a weaker "esterase-lipase" have been detected with practically uniform distribution throughout the gut. The ability of the extracts to hydrolyze beta-naphthyl stearate was so poor that the results are not given. It is unlikely that the amount of enzyme activity recorded is within the limits of accuracy of the method. Oomen (1926), using amyl-acetate and ethyl butyrate esters as substrates, and Sawano (1928) using olive oil, both recorded a weak lipase in *Holothuria* and *Caudina*, respectively. It is unfortunate that there is a considerable degree of overlapping in enzymatic hydrolysis by esterases and lipases even when using purified beta-naphthyl esters as substrates. However, it can be concluded that gut extracts of *Cucumaria* hydrolyze short-chain fatty acids (C_2), and intermediate-chain fatty acids (C_{12}), yet it is doubtful whether they can hydrolyze long-chain fatty acids (C_{18} and upwards).

DISCUSSION

Enriques (1902) suggested that digestive enzymes were carried by amoebocytes from the hemal system into the digestive tract. Oomen (1926) and Schreiber (1930, 1932a, 1932b) found that extracts of the hemal wall contained a protease, invertase, amylase and maltase, yet during his experiments Oomen found that extracts of the stomach wall contained more of these enzymes than did the hemal

TABLE IV

Activity of lipolytic enzymes from the different gut regions

	Esterase	"Esterase-lipase"
Esophagus	4.02 ± 0.4	0.26 ± 0.2
Stomach	3.03 ± 0.5	0.17 ± 0.2
Constriction	3.23 ± 0.4	0.13 ± 0.2
Intestine I	3.78 ± 0.4	0.28 ± 0.3
Intestine II	3.29 ± 0.4	0.16 ± 0.2

Activity expressed as mg. beta-naphthol/ml. of extract/hour. Each value is the mean and standard deviation of 5 determinations.

extract or the digestive fluid. However, the presence of digestive enzymes in an extract of the hemal wall was accepted as more or less verification of the hypothesis of Enriques. According to Frenzel (1892, *vide* Oomen, 1926), the walls of the rete mirabile are glandular, and their secretion is taken up by the amoebocytes and transported *via* the hemal system to the gut. The amoebocytes pass through the gut wall and between the epithelial cells into the gut lumen where they burst to release their contents. Although Hamann (1883) demonstrated gland cells in the stomachs of *Holothuria* and *Leptosynapta,* there are no records relating to the possible secretion of digestive enzymes by cells surrounding the lumen of the gut. The work of earlier authors appears to have been accepted without confirmation by detailed histochemical and physiological studies.

Studies on the digestive enzyme systems in *Cucumaria* have shown that amylase is present only in extracts of the constriction and intestine I, with a peak density in the constriction. Maltase and invertase are present in all gut regions, but there is always an obvious density peak in the constriction. Proteolytic enzymes show their greatest activity in the intestine. In *Cucumaria* there is no rete mirabile, and all parts of the hemal system have the same histological structure (Fish, 1967). If the supply of digestive enzymes is dependent upon the entry into the gut of loaded amoebocytes, then this would seem to suggest two hypotheses as regards the sites of enzyme secretion. Either the enzymes are secreted solely from the walls of the transverse branches, which are the larger channels of the system, or they are secreted in all parts of the hemal system. If the first hypothesis is valid and amoebocytes carry digestive enzymes from the transverse branches of the hemal system into the gut, then to account for the results given above (with particular reference to amylase), there must be some mechanism which ensures that amoebocytes carrying amylase pass only into the constriction and intestine I. If the second hypothesis is true and amoebocytes carry digestive enzymes from any of the hemal channels, then to account for the distribution of amylase, the dorsal and/or ventral hemal sinuses must produce enzymes needed by the particular part of the gut to which the sinus is attached. If this is the case, then the amoebocytes need only pass through the adjacent gut wall. It is suggested that neither of these hypotheses is tenable. The distribution of digestive enzymes can be correlated with the results of histological and histochemical tests applied to the different gut regions of *Cucumaria* (Fish, 1967). Secretory granules and gland cells have been demonstrated in the constriction and the intestine, respectively, and it is from extracts of these regions that the highest degrees of enzyme activity have been recorded. These results and observations lead to the conclusion that cells of the lining epithelium of the gut secrete digestive enzymes. This does not preclude the possibility that amoebocytes carry enzymes. These enzymes may play some part in the nutrition of the whole animal, or their presence may be attributed to the metabolic requirements of the amoebocytes themselves. Useful information would be gained from a detailed study of the enzyme histochemistry of the holothurian digestive tract.

SUMMARY

1. Estimations of proteases, carbohydrases, and lipases have been made on extracts of the different gut regions of *Cucumaria elongata.*

2. An endopeptidase of a trypsin-like nature and several exopeptidases have been detected, all with maximum activity in the intestine. Cellulase and lactase have not been detected, yet maltase and invertase were found in all regions of the gut, and showed their maximum activity in the constriction and intestine I. Amylase was found only in the constriction and intestine I. A strong esterase and a weaker "esterase-lipase" have been detected with practically uniform distribution throughout the gut, yet it is doubtful whether a true lipase, hydrolyzing long-chain fatty acids (C_{18} and upwards), is present.

3. The work of earlier authors dealing with digestive enzymes and the sites of enzyme production in holothurians has been summarized. Results of quantitative estimations of digestive enzymes in *Cucumaria*, coupled with the results of previous histological and histochemical studies, lead to the conclusion that digestive enzymes are secreted by cells bordering the gut lumen.

LITERATURE CITED

Anderson, J. M., 1953. Structure and function in the pyloric caeca of *Asterias forbesi*. *Biol. Bull.*, **105**: 47–61.

Choe, S., 1962. Biology of the Japanese common sea-cucumber, *Stichopus japonicus* Selenka. Fisheries College, Pusan National University.

Davis, N. C., and E. L. Smith, 1955. Assay of proteolytic enzymes. *In*: Methods of Biochemical Analysis. Ed. D. Glick. Vol. 2: 215–257. Interscience Publishers, New York.

Dunn, M. S., and A. Loshakoff, 1936. Quantitative investigations of amino-acids and peptides. I. Quantitative formol titration by means of the glass electrode. *J. Biol. Chem.*, **113**: 359–369.

Enriques, P., 1902. Digestione, circolazione, e assorbimento nelle oloturie. *Arch. Zool. Ital.*, **1**: 1–58.

Fish, J. D., 1967. The digestive system of the holothurian, *Cucumaria elongata*. I. Structure of the gut and hemal system. *Biol. Bull.*, **132**: 337–353.

Hamann, O., 1883. Beiträge zur Histologie der Echinodermen. Die Holothurien. *Zeitschr. wiss. Zool.*, **39**: 145–190.

Nachlas, M. M., and A. M. Seligman, 1949. Evidence for the specificity of esterase and lipase by the use of three chromogenic substrates. *J. Biol. Chem.*, **181**: 343–355.

Oomen, H. A. P. C., 1926. Verdauungsphysiologische Studien an Holothurien. *Pubbl. Staz. Zool. Napoli*, **7**: 215–297.

Pequignat, E., 1966. 'Skin digestion' and epidermal absorption in irregular and regular urchins and their probable relation to the outflow of spherule coelomocytes. *Nature*, **210**: 397–399.

Plimmer, R. H. A., 1920. Practical Organic and Biochemistry (Rev. ed). Longmans, Green Co., London.

Sawano, E., 1928. On the digestive enzymes of *Caudina chilensis* (J. Muller). *Sci. Rep. Tôhoku Univ. (4) Biol.*, **3**: 205–218.

Schreiber, B., 1930. Studi sull' assorbimento intestinale nelle oloturie. *Pubbl. Staz. Zool. Napoli*, **10**: 235–277.

Schreiber, B., 1932a. Pigmenti e secrezioni nel sistema digerente nelle oloturie. *Pubbl. Staz. Zool. Napoli*, **12**: 18–60.

Schreiber, B., 1932b. Experimenti per lo studio dell' assorbimento intestinale nelle oloturie. *Arch. Zool. Ital.*, **16**: 865–870.

Van der Heyde, H. C., 1922. On the physiology of digestion, respiration and excretion in echinoderms. Dissertation, Amsterdam.

ON THE MORPHOLOGY OF THE NEPHRIDIA OF NEREIS LIMNICOLA JOHNSON

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In the past century there have been a number of reports of observations on the nephridia of the polychaetous annelids. These have ranged from passing notes to detailed morphological treatments. Some of these have been concerned with all families of the polychaetes or with general comments (Benham, 1891; Ehlers, 1864-68; and Goodrich, 1895, 1945); some dealt only with the nephridia of the so-called errant forms (Aiyar, 1933; Fage, 1906; and Goodrich, 1897, 1898 and 1900); and most have considered the nephridia of the so-called sedentary worms.

Relatively little information has been published on the detailed morphology of the nephridia of the Nereidae. Goodrich (1893) described the nephridia of *Nereis diversicolor* and found that the nephridial canal could be divided into four regions which differed in distribution of cilia, diameter of the lumen and extent of tubule convolution. Fage (1906) worked on *Perinereis cultrifera*, observing nephridial histology and reporting on the uptake of neutral red by nephridia in living animals. Krishnan (1952) described the nephridial morphology of three nereid species with contrasting salinity tolerances: *Namalycastis indica* (Southern) (euryhaline); *Nereis chilkaensis* Southern (relatively stenohaline, in slightly brackish environments); and *Perinereis nuntia* (Savigny) (stenohaline, in fully marine situations). Krishnan not only described the nephridia of the three genera, but also related the size of the nephridia and the amount of nephridial vascularization to the ability of the polychaetes to tolerate lowered salinities. He concluded that the large size of the nephridia of *N. indica*, as well as their rich supply of blood vessels, represents an adaptation for a euryhaline existence. He suggested that there is a direct excretion of water from blood vessels to the lumen of the nephridial canal and showed that there is a shrinking and collapse of nephridial blood vessels in specimens of *N. indica* which had been acclimatized to full-strength sea water. Finally, the nephridial morphology of *Nereis vexillosa* Grube has been described (Jones, 1957) and it was noted that the nephridial canal of this species is ciliated throughout and that there are three general regions along the length of the canal, based on the lumen diameter [1] and the amount of convolution. In addition, a reconstruction of the nephridial canal was presented.

Nereis limnicola Johnson has recently been used as an experimental animal by a number of workers. The species was originally described (Johnson, 1903) from Lake Merced, a fresh-water lake which has served as a water supply for the city of

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[1] It should be noted that in Figure 8, Jones, 1957, the scale line of the diagram of the nephridial canal of *Nereis vexillosa* should read 100 micra, not 50; further, Figure 6 is reversed, left for right.

San Francisco, California (for a short account of the history of the lake, see Smith, 1958, p. 61). Subsequently, there were no published reports of the species until Smith (1958) re-collected material from Lake Merced for physiological observations.

Hartman (1938) described and recorded (1944) *Neanthes lighti* from Marin and Sonoma Counties, north of San Francisco Bay. Smith (1950) described embryonic development in specimens of this species from the Salinas River, south of San Francisco, near Monterey, and showed that it is a viviparous self-fertilizing hermaphrodite. Later, Smith (1953) studied the distribution of the species along the Salinas River and reported observations on the salinity cycle of the river over a three-year period and the effect of salinity changes on the distribution of the polychaete.

After his re-collection of *Nereis limnicola,* Smith (1959b) compared the type specimens of *Nereis limnicola* Johnson (1903) with specimens of *Neanthes lighti* Hartman (1938) and concluded that *Neanthes lighti* is a junior synonym of *Nereis limnicola. Neanthes lighti* was referred to *Nereis japonica* Izuka (1908) by Edith and Cyril Berkeley (1956, p. 269), who pointed out the close morphological similarities between *Nereis japonica* and *Nereis diversicolor* O. F. Müller. Smith (1958) compared, in considerable detail, specimens of *Nereis limnicola* from California, Washington, and British Columbia, with specimens of *Nereis japonica* from Japan, and specimens of *Nereis diversicolor* from Scotland, England, Denmark, Finland, France, and New Hampshire. Smith presented strong arguments for the separation of these three species, which are reproductively and geographically isolated. Their close morphological and ecological similarities are emphasized by Pettibone (1963, pp. 160–161) who referred all three species to *Nereis (Hediste).* Hartman (1960) referred *N. limnicola, N. lighti* and *N. japonica* to *Neanthes diversicolor* and Imajima and Hartman (1964) referred *Nereis japonica* Izuka to *Neanthes diversicolor.* However, I prefer to follow Smith (1958, 1959b), Khlebovich (1963), and Pettibone (1963) in considering the three species, *N. diversicolor* O. F. Müller, *N. japonica* Izuku, and *N. limnicola* Johnson, as distinct, but closely related, species of *Nereis (Hediste).*

Nereis limnicola has been utilized by Smith for physiological studies (1957, 1959a), who found that the species can control the influx of pond water, distilled water, and extreme dilutions of sea water at 13° C., but has no control at temperatures of 1°–2° C. Later, Smith (1963), in comparing *N. diversicolor, N. limnicola,* and *Nereis (Neanthes) succinea,* found that *N. limnicola* had the lowest salt loss rate of the three species when placed in lowered salinities, but (Smith, 1964) that both *N. limnicola* and *N. succinea* have an equal D_2O influx at a body weight of about 100 mg., even though *N. limnicola* takes up less water when both species are subjected to an equal external osmotic gradient.

Stephens (1964) made observations of the uptake of glycine by *N. limnicola* and *N. succinea.* He found that the uptake by the latter is greater by an order of magnitude than in the former and suggested that the uptake takes place across the body wall. Stephens further suggested that glycine uptake and osmoregulation are incompatible, since glycine uptake becomes less, and even ceases, when the salinity of the medium is lowered into the range wherein the worms are hyper-regulating.

Oglesby (1965a), in comparing water and chloride regulation in *N. limnicola,* *N. succinea*, *N. vexillosa*, and *Laeonereis culveri* (Webster), reported that *N. limnicola* shows the best ability to regulate osmotic concentration and exhibits the least change in water content of the entire body with varying salinities. Further, Oglesby (1965b) has shown that the chloride exchange rate is lowest in *N. limnicola* and suggested that this may be due to a low chloride permeability, the worms becoming essentially impermeable to chloride in fresh water.

In his paper treating of viviparity in *Nereis limnicola*, Smith (1950) dealt with worms inhabiting the lower Salinas River, Monterey County, in central California. The Salinas River presents a difficult situation for aquatic forms. It is not a large river and appears to serve mainly as a run-off channel for the fall, winter, and spring rains from its watershed. After the spring run-off, a sandbar is formed across the mouth of the river, forming a "blind" estuary of the river, as defined by Day (1951). This serves to dam the river flow until the following winter when the press of run-off may be sufficient to break through to the sea. The latter is not necessarily an annual event and the river mouth may remain blocked for several years.

Smith's studies (1950) were concerned with the worms found in two areas. One ("Area A" on Smith's map, p. 425) was a "muddy channel in a *Salicornia* marsh near the mouth of the Salinas River," and the other (Area B) was "a sandy stretch of river about four miles upstream, where the general aspect is that of fresh water." Smith further gave the range of salinities for these areas as 20% to 115% sea water for Area A and 1% to 3% sea water for Area B, the salinity of a given area being a function of rainfall and season. He pointed out that the effect of the more extreme low salinities was possibly damped by the residual salt in the soil of the surrounding substrate. Smith (1953) later provided more detailed ecological observations on the lower Salinas River and referred to seven numbered locations (location 3 = Area A, above; location 4 = Area B).

During the rainy season and the period of run-off, the *Salicornia* marshes of the lower Salinas River may be flooded with fresh water. Within a few days the salinity of the overlying water is decreased immensely. This dilution prevails for a variable period until the last of the seasonal excess is sluiced into the sea. At this time, as the fresh water recedes, the marshes may be inundated with sea water at spring tides. As the flow of the river decreases, the channels in the marsh become isolated from the main river. Through the summer, isolated ponds are subjected to the evaporative effect of the sun, although they are relieved by sporadic rains and heavy fogs. During this time, the salinity of the overlying water reaches its annual high that is maintained until the fall rains, when the rising river inundates the isolated areas and dilutes them to their annual low. In the descriptions and discussion to follow, the worms of Area A will be referred to as the down-river population.

Throughout the year there is near-fresh water over Area B, several miles up-river from Area A. This condition is relieved only during times of extremely high tides when the river flow is slackening, following the winter rains, at which times incursions of salt water may reach Area B. The worms found in this area are probably near the extreme fresh-water end of their range, for Smith (1953, location 5), has not found them more than $\frac{1}{2}$ mile up-stream from the collection site in Area B. The worms of Area B will be referred to as the up-river population.

Considering the brief ecological description above, several questions become apparent. Is it reasonable to expect that groups within the same species, differing only in the salinity of their environments, will exhibit morphological differences? Or, because of a physiological adaptability, will these groups show no significant anatomical differences? If there are differences, will they be manifest in the nephridium? It was in an attempt to answer these questions that this morphological comparison of the nephridia of *Nereis limnicola* from environments of different salinity was undertaken.

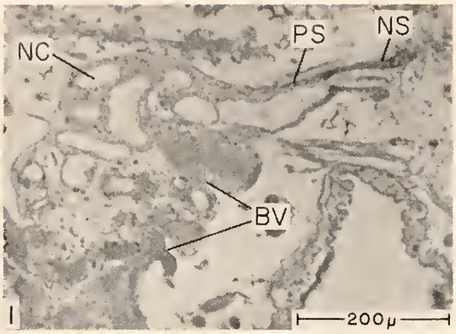
THE NEPHRIDIUM OF NEREIS LIMNICOLA (UP-RIVER FORM)

The worms used in the following section were collected in Area B (Smith, 1950). The salinities of the water flowing over them were less than 2.5% sea water. The worms were relaxed by the use of dilute alcohol, fixed in Bouin's, serially sectioned in paraffin at 6 micra, stained in Harris' hematoxylin, and counterstained in eosin.

Within the same animal, indeed, within the same segment, there may be a wide variation in the overall size of the nephridia. In one case, a pair of nephridia in the same segment were observed in which the sizes differed in the order of 1:2. These approximated the extreme differences between pairs of nephridia, as well as between unpaired nephridia. They were both approximately the same width and length (250 micra), but differed, however, in that one was about 240 micra in height while the other was about 400 micra (for lengths and numbers of segments of the worms examined, see Table II). The approximate volume of the former was 0.0106 mm.^{3} and the latter, 0.0140 mm.^{3}

If one views the nephridium of *Nereis limnicola* from Area B, with the purpose of comparing it to that of *Nereis vexillosa* (Jones, 1957), one is immediately impressed by the vast number of blood vessels in contact with, and buried in, the tissue of the nephridium (Figs. 1, 2, and 3, BV). The shape of the nephridium also contrasts with that of *N. vexillosa.* Whereas the nephridium of *N. vexillosa* is globular and possesses a smooth, even surface, that of *N. limnicola* shows a suggestion of a division into regions. The dorsal half of the nephridium is oval in cross-section, and is somewhat compressed antero-posteriorly. The ventral half is nearly circular in cross-section, and is more or less hemispherical. At the equator of the hemisphere, the post-septal canal enters the nephridial mass in company with the ventral segmental vessel, that ramifies over the surface of the nephridium (Fig. 1, PS). At the point of entry of the post-septal canal there is a slight swelling. The surface of the nephridium shows a slight indication of the internal canal in the more vascularized portion, while the other half of the nephridium externally shows a well-defined canal.

The sectioned nephridium of the up-river form of *Nereis limnicola* shows much the same aspect as *Nereis vexillosa* (Figs. 1, 2, and 3), and a number of nuclei are scattered throughout the sectioned area. Further, there are occasional areas of vacuolation, but not to the extent of those observed in *N. vexillosa.* On the whole, the perforations or sections of tubule lumen observed in the sections of the nephridia of the up-river form of *N. limnicola* presented the same appearance as those of *N. vexillosa.*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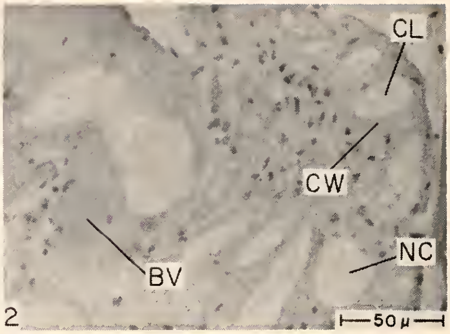
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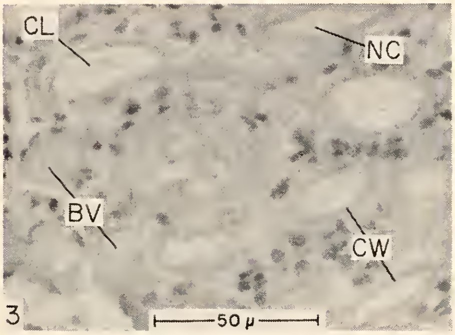
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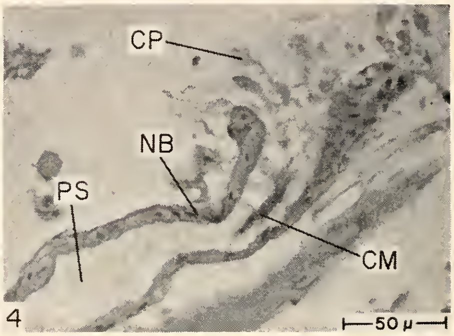
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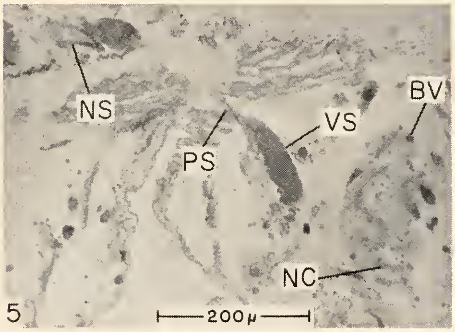
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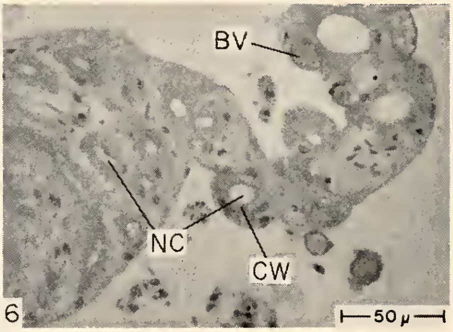
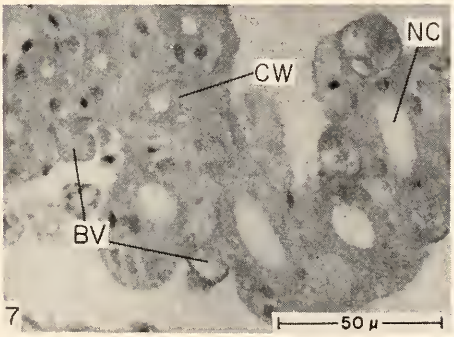
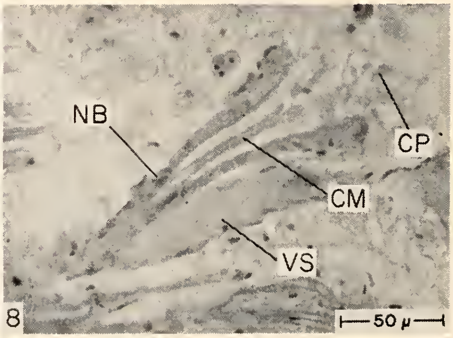
BV
VS
PS
NC
5
200 μ


BV
NC
CW
6
50 μ


NC
CW
BV
7
50 μ


NB
CP
CM
VS
8
50 μ

The ciliation of the nephridial canal (Figs. 2 and 3, CL), as noted in this form, did not seem to differ significantly from the pattern seen in *N. vexillosa*. Cilia were noted throughout the length of the canal, from the nephrostome to within 40 to 50 micra of the nephridiopore. As before, there seemed to be no distinct division in the nephridial canal on the basis of its ciliation.

As mentioned above, the walls of the tubules are only occasionally distinct. When present, they consist of vacuolated areas around the periphery of the perforation. They give the appearance of a clear ring around the lumen, and may have some intradivision in the form of faint, thin walls. In the "non-walled" perforation, the fine network of the interstitial tissue comes up to the canal boundary, and no basement membrane is visible. In all probability, there actually is or was a wall present, but staining and/or fixation techniques may not have been adequate to bring it out. A variation of this last type of wall occurs when the area immediately surrounding the perforation appears to be more heavily stained than the adjacent interstitial tissue (Figs. 2 and 3, CW). By careful examination, it is seen that this darkening is due to the presence of a more concentrated net system and many granular inclusions.

In the nephridium of *Nereis vexillosa* it was noted that blood vessels were at a minimum, approaching and possibly contacting the nephridial system at only two points. In *Nereis limnicola* from the up-river area, it is readily seen that the nephridium is penetrated throughout by many vessels. In the main, they are confined to the more peripheral areas, but many branches pass through the center of the mass (Figs. 1, 2 and 3, BV). The ventral segmental vessel, after it approaches the nephridial mass in company with the post-septal canal, ramifies over the lateral face of the nephridium and at several points passes dorsally into the interior. The ventral portion of the nephridium has no internal blood vessels, while the dorsal half contains more vessels than it carries on its surface. Occasionally, there are blood vessels on the surface of the nephridial mass which seem to have sunken into the tissue. They are not surrounded by nephridial tissue, but are in close contact with it over about 180° to 200° of their circumference in section.

In contrast to the long post-septal canal of *Nereis vexillosa*, this structure in the up-river form of *Nereis limnicola* is extremely short (Fig. 1, PS, and Fig. 9A), the length of the former being 250 micra, and that of the latter about 175 micra.

Key to lettering: BV, blood vessel; CL, cilia; CP, cytoplasmic processes of nephrostome; CM, mass of cilia; CW, nephridial canal wall; NB, band of nuclei of nephrostome (= septal band); NC, nephridial canal; NS, nephrostome; PS, post-septal canal; VS, ventral segmental vessel.

Figures 1–4, up-river form of *Nereis limnicola*; Figures 5–8, down-river form of *N. limnicola*.

FIGURE 1. Dorsal view of right nephridium and associated nephrostome; specimen RB.

FIGURE 2. View of nephridial tissue; specimen S-2.

FIGURE 3. Detailed view of nephridial tissue and associated blood vessels; specimen S-2.

FIGURE 4. Nephrostome; specimen RB.

FIGURE 5. Dorsal view of left nephridium; specimen SB.

FIGURE 6. View of nephridial tissue at junction of medial (left) and lateral (right) regions; specimen S-3.

FIGURE 7. Detailed view of nephridial tissue and associated blood vessels; specimen S-3.

FIGURE 8. Nephrostome; specimen S-3.

Further, the diameter of this portion of the nephridial canal is slightly larger in the up-river form of *N. limnicola*. Proceeding through the post-septal canal from the nephridial mass toward the nephrostome, the cross-sections of the isolated canal show the same structure noted in *N. vexillosa*. The wall appears to be vacuolated, with occasional larger nuclei. Through the proximal portion of the post-septal canal, the nuclei are well-scattered along the proximal portion of the post-septal canal, but become more concentrated toward the middle portion, where there are about twelve visible in each section (6 micra thick). Throughout this part of the canal, cilia are visible, distributed around the inner boundary of the wall. The diameter of the lumen is about 15 micra, basally, near the nephridial mass, and distally narrows to about 7 micra. These conditions prevail throughout the proximal 100 micra of the post-septal canal. Where the lumen is at its narrowest, immediately distal to the region just described, and at about the level of the passage of the post-septal canal through the septum, the nuclei within the walls are quite concentrated; 28 to 30 are distributed fairly evenly around the lumen in a section. This concentration of nuclei, in what might be called a "septal band" (Fig. 4, NB), is not so great nor so extensive as in *N. vexillosa*; a comparison shows a more restricted area in *N. limnicola*.

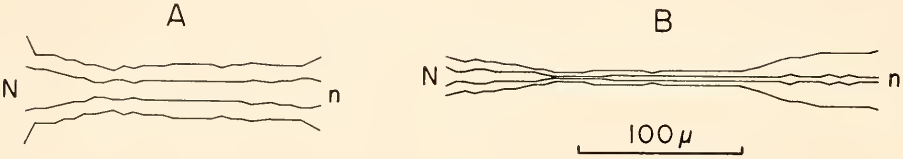


FIGURE 9. Graphic representation of post-septal canals associated with nephridia of *Nereis limnicola*. A. Up-river form; specimen RB. B. Down-river form; specimen SB. N, nephrostome; n, nephridial mass.

Immediately anterior to the narrowing of the canal and the nuclear concentration, the canal widens to form the nephrostome, its extreme width being 25 to 30 micra at its mouth. All along the walls of the funnel the nuclei are scattered evenly, as they were at the beginning of the post-septal canal. As in *N. vexillosa*, the lumen of the nephrostome is almost choked by the tangle of cilia lining it (Fig. 4, CM). Many fairly large club-like structures, the protoplasmic processes, occur at the opening of the nephrostome (Fig. 4, CP). In some of these are found the same type of inclusion that appears in the interstitial tissue and tubule walls of the nephridial mass. It is difficult to make out the exact structure of the processes, for they stain weakly, and, at times, are intermeshed with the cilia that originate in the walls of the nephrostome and the bases of the processes. From the tops and sides of most of the processes, long cilia project into the open mouth of the funnel. It is to be noted that the processes in this species are different from those found in *N. vexillosa*; in *N. vexillosa*, they are long and thin, while in *N. limnicola*, they are stout and nearly pyriform.

Figure 10 shows the diameter of the canal of the up-river form of *Nereis limnicola* from the entrance of the post-septal canal into the nephridium, to the nephridiopore (the canal chosen was the largest of the nephridia observed in detail).

The canal has an overall length of 2232 micra and a mean diameter of 18.9 micra (the length of the canal of the smallest nephridium was found to be 1800 micra and its mean diameter was 19.6 micra). There appear to be four different regions with respect to lumen diameter. The first, diameter about 24 to 30 micra, extends from the entrance of the post-septal canal for about 800 micra; the second, whose diameter is in the range of 36 to 40 micra, extends for another 600 micra; the third, the narrowest part, about 12 to 27 micra, runs for approximately 200 micra before grading into the final portion; the last portion, about 750 micra in length, increases from 27 to 48 micra, then decreases somewhat irregularly until it reaches the nephridiopore, where its diameter is 6 micra. The sudden widening of the canal just prior to the nephridiopore gives the appearance of an ampulla.

In the last portion of the nephridial canal, as the nephridiopore is approached, the wall of the lumen seems to become thicker and more dense. Closer inspection shows that the network of the interstitial tissue has become more concentrated in the immediate area of the canal and that there seems to be an increase in the number of granular inclusions contained in this net. There are large nuclei scattered through the wall of the canal, until, at a point about 40 micra from the external opening, a more regular distribution is assumed, with three or four nuclei apparently in the same plane. This continues to the last 8 micra, where large nuclei are clustered around the canal, and the wall loses its identity in the surrounding tissue. As was the case in *N. vexillosa,* there is no ciliation in the canal through the last 40 to 50 micra. Though an ampulla was not so obvious in *N. vexillosa, N. limnicola* usually shows an ampulla (Fig. 10), or a suppression of one, just interior to the nephridiopore. In cases of suppression, the lumen as seen in section is tripartite, with the walls pressed together until the lumen cross-sectional area is at a minimum. Whether this is an artifact of fixation, a morphological anomaly, a sphincter-like device for closing the canal, or an adaptation providing a greater surface-volume ratio for more efficient resorption or excretion, is not clear, but, as this type of structure was fairly common, the condition seems most probably related to resorption-excretion or to canal closure.

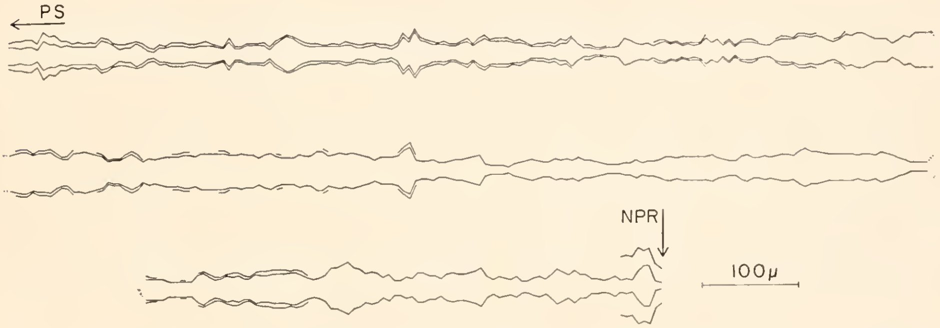


FIGURE 10. Graphic representation of the inner diameter and wall thickness of a nephridial canal of *Nereis limnicola* from up-river (reconstructed from sectioned material). PSC, post-septal canal; NPR, nephridiopore.

The Nephridium of Nereis limnicola (Down-River Form)

The worms used as a basis for the following description were collected at Area A (Smith, 1950). The salinity of the water standing over them was at least 47.5% sea water. (It is necessary to point out that one of the worms, S-3, was obtained from Dr. Ralph I. Smith, who had adapted it from 81% sea water to 106%. It is assumed that consideration of this worm is not remiss, for this salinity is well within the range reported for the species and at none of these salinities are the worms osmoregulating.) As before, the worms were relaxed, fixed in Bouin's, sectioned serially at 6 micra, stained in Harris' hematoxylin, and counterstained with eosin.

One of the most obvious characteristics of the nephridia of the down-river form of *Nereis limnicola* is their shape. Whereas in the up-river form there was a slight compression in the dorsal portion and the ventral half was hemispherical, in the worms from the *Salicornia* marsh, there is a general compression of the entire nephridium (Fig. 5). This usually is seen to occur parallel to the axis of the post-septal canal which projects obliquely, anteriad and mediad. In extreme dimensions, the nephridium measures about 400 micra long, 350 micra high, and 150 micra thick (through the medial half). The lateral half is approximately 50 micra thick, and the approximate volume is 0.0122 mm.^{3}

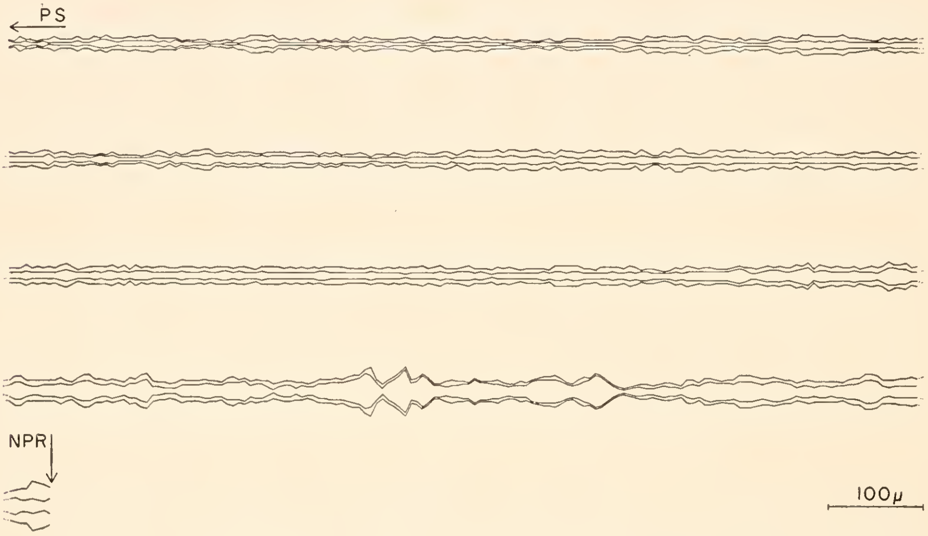


FIGURE 11. Graphic representation of the inner diameter and wall thickness of a nephridial canal of *Nereis limnicola* from down-river (reconstructed from sectioned material). PSC, post-septal canal; NPR, nephridiopore.

As just intimated, there is an external division into medial and lateral halves, the medial half being elliptical in cross-section, and the lateral half being extremely compressed to about one-third the thickness of the other. In the extreme dorsal sections of the nephridium the two halves are entirely separate.

In comparing the sectioned nephridia of the down-river and the up-river forms of *N. limnicola,* the first glance at those from down-river would lead one to doubt that the two were at all related. A considerable reduction occurs in the diameters of the canal lumen, and, to a lesser degree, a reduction in the number of blood vessels (Fig. 5). In addition, the interstitial tissue seems more dense than that in either the up-river form or in *N. vexillosa*.

The diameter of the tubule lumen is, in most portions, as little as 1 to 2 micra. In some cases, the lumen of the canal is almost completely closed and only a pinpoint of clear area is visible by careful focusing. Under these circumstances no measurement is possible, and in graphing the tubule diameter (Fig. 11), these perforations were considered to be one micron or less in diameter. At other times, the perforations were obscured either by heavy ciliation or a turning of the canal within the 6-micron thickness of the section. It is possible that this general narrowness of the canal might have been an artifact caused by osmotic factors during fixation and/or relaxation. However, there were portions of the tubule present in the same section, with diameters comparable to those of *N. vexillosa* and the up-river form of *N. limnicola* (Figs. 6 and 7). In some, a well-defined boundary was visible, complete with a basement membrane separating the tubule wall from the interstitial tissue. In others, there was an irregular area of vacuolation surrounding the perforation. In still others, the network of the interstitial tissue extended up to the canal and the poorly-defined wall may have been due to a thickening of the network or to the presence of extremely fine particulate material. It was impossible to make an accurate judgment here, for the nature of the darkening was not resolvable, microscopically. It is interesting to note in these worms from down-river, that not all of the peripheral areas of the nephridial perforations were well-defined, but that all were surrounded by an area that stained darker with hematoxylin than the interstitial tissue. Indeed, in the case of those lumen perforations which were closed most tightly, the darkened areas helped to locate the fine canal openings.

As stated above, the down-stream form stands in contrast to its up-stream counterpart in the lesser amount of vascularization of the nephridium. Of the two nephridial halves referred to, the more lateral is the more vascularized (Fig. 6, BV). The nephridium of the down-river form is extremely well-supplied with a network of small blood vessels that ramify over its surface (especially that of the lateral half). These find their origin in the ventral segmental blood vessel, which itself proceeds over the anterior face of the medial half of the nephridium, and finally departs toward the parapodium, about 60 micra from the body wall. In the central part of the nephridium, this vessel gives rise to a branch that remains in contact with the medial half until immediately before ventral contact is made with the body wall (Fig. 5, VS). This last branch and the large ventral segmental vessel are the only blood vessels in contact with the medial half of the nephridium.

In the up-river form, the post-septal canal is fairly short and has a lumen diameter much the same as that of the main canal in the nephridial mass; in the down-river form (worms of comparable size) the post-septal canal is twice as long (about 250 micra) and the lumen is almost entirely closed at many points (Fig. 9B). The post-septal canal wall of the down-river form is also much thinner, about 1 to 2 micra for the most part. The small size of the tubule makes it difficult to trace from the nephridial mass to the nephrostome, for it is closely applied to the ventral segmental vessel throughout (Fig. 8), and at times, in transverse section, resembles a small cell attached to the blood vessel. The nuclei which are visible within the tube wall possess little chromatin. The "septal band" separating the post-septal canal from the nephrostome is composed of a concentration of nuclei, but is not so extensive as the bands previously described for the up-river form and *N. vexillosa* (Fig. 8, NB).

The nephrostome (Fig. 8), which extends anteriorly about 100 micra from the dark band, has walls that appear to be solid, and there are no large vacuoles within them. The walls seem to be about the same density as the interstitial tissue of the nephridial mass. Around the margin of the nephrostome, the protoplasmic processes are stout, club-shaped structures that give rise to long cilia (Fig. 8, CP). Their shape would seem to bear out Goodrich's (1945) statement concerning the specificity of these structures, for they are similar to those observed in the up-river form, but differ from those of *Nereis vexillosa* and *N. diversicolor*. As before, the number of cilia originating inside the funnel is sufficient to clog the lumen (Fig. 8, CM).

Figure 11 shows the diameter of the nephridial lumen of the down-river form of *Nereis limnicola*. It is seen that the lumen is quite narrow at its beginning (of the order of 1 to 6 micra) and gradually increases in size, until at the three-quarter mark, it is consistently larger. Beyond this point, it undergoes a series of irregularities, grows extremely wide, closes once more, and finally becomes fairly uniform close to the nephridiopore. It is fully walled throughout; at, and just subsequent to, its widest part, the wall is at its thinnest; also, the wall thickens considerably as it approaches the nephridiopore. In the case of the nephridium upon which the diagram is based, the length of the canal within the nephridial mass is about 3864 micra, with a mean diameter of 9.3 micra.

The region of the nephridiopore of the down-river form of *N. limnicola* is essentially the same as that of the up-river form. As the nephridial canal approaches the body wall, the walls of the canal thicken, and contain large, relatively clear, nuclei. At times, the area shows the same compression as described for the up-river form.

Discussion

Several points emerge from the descriptions above: the nephridia of both the up-river and down-river forms of *N. limnicola* are more highly vascularized than those of *N. vexillosa;* the nephridia of the up-river form are more highly vascularized than those of the down-river form; the down-river form possesses a longer and more narrow nephridial canal than the specimens from up-river; and the nephridial blood vessels of both forms do not come into contact with the nephridial canal.

Krishnan (1952) found that the nephridia of *Namalycastis indica,* a euryhaline species, were larger and more heavily vascularized than those of the other nereids he studied. He also found that some of the nephridial blood vessels were in intimate contact with the canal wall and that, in the case of worms acclimatized to full-strength sea water, there was a lessening of the blood supply to the nephridia, in terms of shrunken and collapsed vessels. He suggested (p. 248) that the reduced blood supply might indicate that these nephridia ". . . are probably doing less osmotic work than in the normal forms living in fresh water." Krishnan further postulated that there is a direct relationship between the size of nephridia and the osmoregulatory ability of the species in question, and that the ability of a nereid to osmoregulate also was reflected, not only by the amount of nephridial vascularization, but by the proximity of blood vessels to the nephridial canal.

It would seem from the series of three species considered by Krishnan that there is, indeed, a correlation between nephridial size and the ability to osmoregulate; but it should be noted that *Nereis vexillosa,* a stenohaline, relatively high-salinity species, possesses nephridia nearly as large as those of *Namalycastis indica* (Jones, 1957). Further, the nephridia of the up-river form of *Nereis limnicola,* which one would assume to be osmoregulating, are larger than those of *N. indica,* but quite a bit smaller than those of the down-river form which one would assume to be doing less osmoregulatory work.

TABLE I

Derivation of Indices of Excretory Capacity of nephridia from specimens of Nereis limnicola, from up-river (S-2), adapted from low to high salanity (S-1), from down-river (S-3), adapted from high to low salinity (S-4)

	A	B	C	D	E	F
Worm	Number of sections counted	Assumed total number of canal sections (A × 3)	Assumed length of canal (B × 6 μ)	Number of segments	Length (μ)	Index of Excretory Capacity $\left(\frac{C \times 2D}{E}\right)$
S-2	128	384	2304	50	38,000	6.982
	100	300	1800	50	38,000	5.455
	98	294	1764	50	38,000	5.345
	86	258	1548	50	38,000	4.691
S-1	237	711	4266	42	30,000	11.945
	194	582	3492	42	30,000	9.778
S-3	290	870	5220	61	35,000	18.191
	274	822	4932	61	35,000	17.192
	224	672	4032	61	35,000	14.054
	252	756	4536	61	35,000	15.811
S-4	243	729	4374	62	33,000	16.436
	194	582	3492	62	33,000	13.121

Clearly, some character other than size, alone, allows these various nereids to survive in a dilute medium. Krishnamoorthi (1963b, 1963c) invoked size as a criterion of regulatory ability but, in addition, suggested that the length of the nephridial canal, as embodied in his "Index of Excretory Capacity" (= length of excretory surface, in microns/length of worm, in microns; "excretory surface" is defined as the average length of nephridial canal multiplied by the average number of nephridia per worm), was also a reflection of osmoregulation. Krishnamoorthi found that the indices of excretory capability were correlated with the distribution of four polychaetes, as he found them in the River Adyar and the nearby Bay of Bengal (Krishnamoorthi, 1963a): *Diopatra variabilis* Southern, index = 0.350, salinity range = 20–26‰; *Euclymene insecta* (Ehlers), 0.310, 20–26‰; *Onuphis eremita* Audoin and Milne Edwards, 0.247, 30–34‰; and *Loimia medusa* (Savigny), 0.225, 30–34‰. Although an extended series of pertinent observations was not conducted on the length of nephridial canals of the up-river and down-river forms of *N. limnicola*, certain assumptions can be made. If one assumes that the number of canal sections counted in every third nephridial section (Table I, column A) is a reasonable estimate of one-third of the total number of canal sections, then, by multiplying by three (Table I, column B) and by the thickness of the sections, 6 microns (Table I, column C), one can arrive at an estimate of the length of a given nephridial canal. If this number is multiplied by twice the number of segments of the worm and this is, in turn, divided by the worm's length, in microns, one obtains Krishnamoorthi's Index of Excretory Capacity. Depending upon which nephridium and which population is chosen, the indices vary from 4.691–6.982 for the up-river forms to 14.054–18.195 for the down-river forms (Table I, column F). Worms cross-adapted from high to low and from low to high salinities give intermediate indices.

It would seem, intuitively, that the up-river population would have need of a greater "excretory capability," yet it has the lowest indices of the specimens of *N. limnicola* considered here. In addition, the lowest of the index values are more than ten times those found by Krishnamoorthi. Clearly, then, the Index of Excretory Capacity, itself, can not give an adequate idea of the osmoregulatory capabilities of a polychaete living in a low-salinity or fresh-water habitat.

Krishnan (1952) and Krishnamoorthi (1963b, 1963c) also have suggested that there is a correlation between the amount of vascularization and the ability to osmoregulate. Although subjective observations of the amount of nephridial vascularization of *N. limnicola* would seem to confirm this, I have not found a satisfactory method of quantifying these differences.

Yet another nephridial parameter might be considered, in addition to overall nephridial size, relative length of nephridial canal, and nephridial vascularization. Reduced to essentials, the survival of an animal with a permeable integument in a hyposmotic medium depends on (a) its ability to control its volume and, in effect, to slow or stop the osmotic inflow by hydrostatic pressure; (b) its ability to tolerate a dilution of its body fluids; or (c) its ability to counteract the dilutive effect of the osmotic inflow by the rapid excretion of water. Although the first two possibilities are outside the purview of the present work, observations have been made above, which bear on the third.

A number of papers have appeared which have been concerned with various physiological responses of *N. limnicola* to dilute or fresh water media. All of these postulate that there must be some means of volume control (Smith, 1963), a means of modifying salt loss rate (Smith, 1963), and/or a means of increasing the ability of the worm to eliminate excess water (Smith, 1959a, 1963; Oglesby, 1965b).

It has been noted that there is an apparent difference in the diameter of the nephridial canal of the two forms of *N. limnicola* considered here. In an effort to establish the statistical validity of these apparent differences, a number of nephridia of both forms were examined (Table II). Using 14 nephridia from six different up-river specimens and ten nephridia from four different specimens from the down-river area, all of the perforations of sectioned nephridial canals were measured in every third section of each nephridium. The results of all measurements of all nephridial canals of both forms were cast as frequency distributions (Fig. 12), and it was found that the mean canal diameter of the up-river forms

TABLE II

Collection data and various measurements of specimens of Nereis limnicola considered in the present study

Worm	Collection data		Salinity at death	Body segments	Length mm.	Mean canal diameter (μ)	Mean ± 2 S.E. (μ)
	Date	Salinity					
Nereis limnicola, Up-river							
S-2	10 Dec. 1950	0.55% SW	0.75% SW	50	33	22.84	21.28–24.40
S-2	10 Dec. 1950	0.55% SW	0.75% SW	50	33	16.82	15.64–18.00
S-2	10 Dec. 1950	0.55% SW	0.75% SW	50	33	11.03	10.03–12.03
S-2	10 Dec. 1950	0.55% SW	0.75% SW	50	33	10.66	9.66–11.66
RB	6 May 1951	2.45% SW	2.45% SW	52	—	30.36	28.18–32.54
RB	6 May 1951	2.45% SW	2.45% SW	52	—	21.59	19.63–23.55
S-10	6 May 1951	2.45% SW	2.45% SW	46	—	19.27	18.09–20.45
S-10	6 May 1951	2.45% SW	2.45% SW	46	—	17.74	16.60–18.88
49C	13 Jan. 1949	1.49% SW	1.49% SW	—	—	31.87	30.57–33.17
49C	13 Jan. 1949	1.49% SW	1.49% SW	—	—	27.26	25.08–29.44
49D	13 Jan. 1949	1.49% SW	1.49% SW	—	—	20.07	18.23–21.91
49D	13 Jan. 1949	1.49% SW	1.49% SW	—	—	18.51	16.27–20.75
49E	13 Jan. 1949	1.49% SW	1.49% SW	—	—	8.59	7.55– 9.63
49E	13 Jan. 1949	1.49% SW	1.49% SW	—	—	9.21	8.07–10.35
All data pooled	—	—	—	—	—	20.79	20.48–21.10
Nereis limnicola, Down-river							
S-3	10 Dec. 1950	81.00% SW	106.00% SW	61	35	9.79	8.97–10.61
S-3	10 Dec. 1950	81.00% SW	106.00% SW	61	35	9.54	8.72–10.36
S-3	10 Dec. 1950	81.00% SW	106.00% SW	61	35	9.09	8.37– 9.81
S-3	10 Dec. 1950	81.00% SW	106.00% SW	61	35	8.83	8.15– 9.51
SB	6 May 1951	48.00% SW	48.00% SW	64	—	4.83	4.23– 5.43
SB	6 May 1951	48.00% SW	48.00% SW	64	—	4.18	3.74– 4.62
S-13	6 May 1951	48.00% SW	48.00% SW	68	—	4.63	4.07– 5.19
S-13	6 May 1951	48.00% SW	48.00% SW	68	—	4.33	3.89– 4.77
51A	21 Feb. 1951	47.50% SW	47.50% SW	57	—	11.21	10.11–12.31
51A	21 Feb. 1951	47.50% SW	47.50% SW	57	—	10.88	9.64–12.12
All data pooled	—	—	—	—	—	8.49	8.19– 8.79
Nereis limnicola, Cross-adapted							
S-1	29 Apr. 1951	0.55% SW	118.00% SW	42	30	22.19	20.85–23.53
S-1	29 Apr. 1951	0.55% SW	118.00% SW	42	30	11.89	11.17–12.61
S-4	1 June 1951	81.00% SW	0.80% SW	62	33	17.32	16.40–18.24
S-4	1 June 1951	81.00% SW	0.80% SW	62	33	13.58	12.82–14.34
Nereis vexillosa, San Francisco Bay							
V1	3 May 1951	73–90% SW	73–90% SW	—	—	16.92	15.56–18.28
V1	3 May 1951	73–90% SW	73–90% SW	—	—	16.75	15.53–17.97
V2	3 May 1951	73–90% SW	73–90% SW	—	—	14.17	13.13–15.21
V2	3 May 1951	73–90% SW	73–90% SW	—	—	15.11	13.67–16.55
V4	3 May 1951	73–90% SW	73–90% SW	—	—	15.67	14.01–17.33
V4	3 May 1951	73–90% SW	73–90% SW	—	—	15.73	14.33–17.13

was 20.79 micra (one standard error = 0.31 micra) and that of the down-river forms was 8.49 micra (one standard error = 0.15 micra). Utilizing the "Student" t test, it was found that there was, indeed, a significant difference between the mean canal diameters of the two forms ($t = 38.44$). This also can be interpreted as the difference between the two means being 38.44 times the standard error of this difference.

The results above, however, may not be so straightforward as they might seem. If the mean canal diameter (± 2 standard errors) of each nephridium examined is plotted against salinity (Fig. 13), it is seen that there is a rather large spread of the data derived from the up-river forms. Indeed, the results from three of the up-river worms (S-2, 49C and RB) indicate that there is a real difference between and among the diameters of the nephridial canal in the same animal, and two specimens (S-2 and 49E) have nephridial canals whose diameters are not significantly different from at least some of those from the down-river locality.

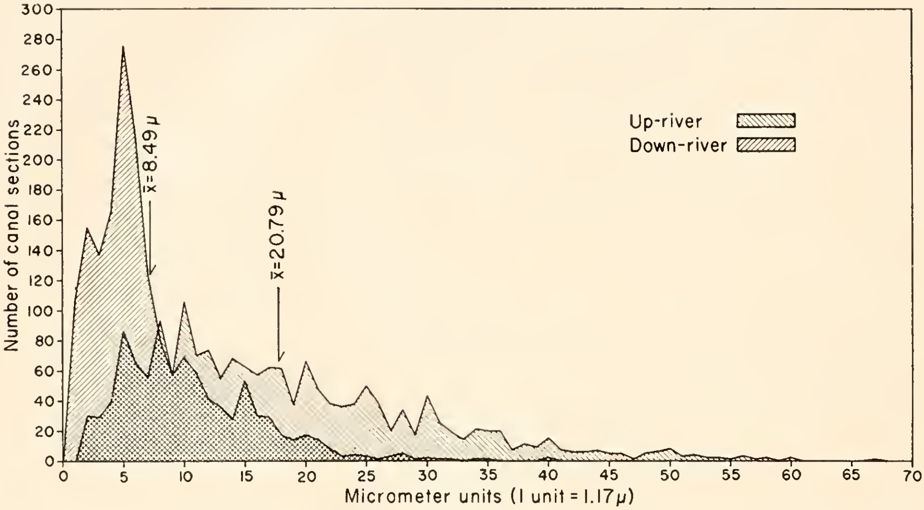


FIGURE 12. Frequency polygons showing the difference in nephridial canal diameter between the up-river forms of *Nereis limnicola* (based on 14 nephridia from six specimens) and the down-river forms (based on ten nephridia from four specimens).

In addition to observations of the nephridia of worms sacrificed directly from the salinities in which they were collected, examinations were made of the nephridia of two cross-adapted worms. In the case of specimen S-1 (originally up-river), the adaptation was from 0.55% sea water to 118% sea water and of S-4 (originally down-river), from 81% sea water to 0.80% sea water. The general aspect of the nephridia of both S-1 (Figs. 14 and 15) and S-4 (Figs. 16 and 17) is strikingly similar to the nephridia of the up-river population of *Nereis limnicola.* The average canal diameter of S-4 (Fig. 13) falls among the lower values of the up-river forms, while that of S-1 is comparable to the larger canal diameters of the up-river population, even though S-1 was acclimatized to 118% sea water just before it was sacrificed.

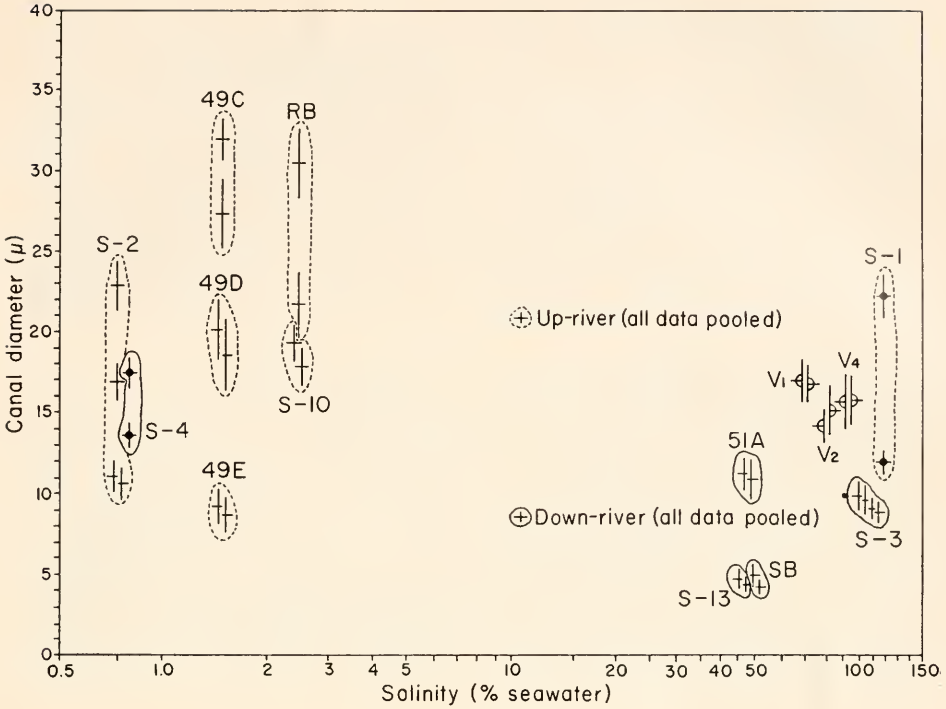
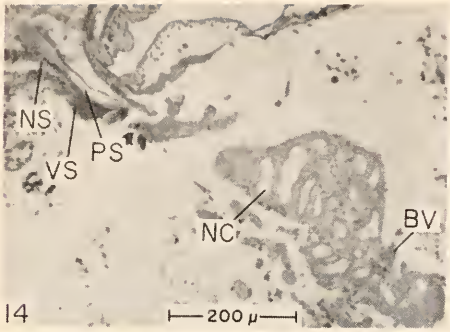
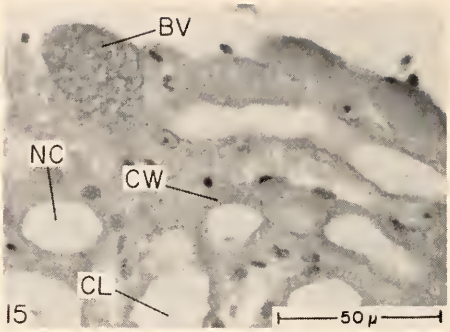


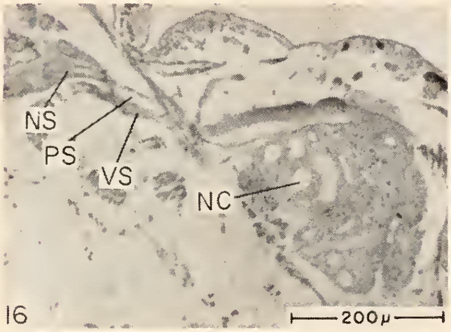
FIGURE 13. Graph showing the relationships among all nephridial canals considered. Horizontal lines represent mean canal diameters for each nephridium examined and the vertical lines, two standard errors above and below the mean. Specimen numbers are referable to Table II. Symbols surrounded by solid lines represent down-river forms; those with dashed lines, up-river forms; those with central solid circles, cross-adapted specimens; and those with central open half-circles, *Nereis vexillosa* from San Francisco Bay.

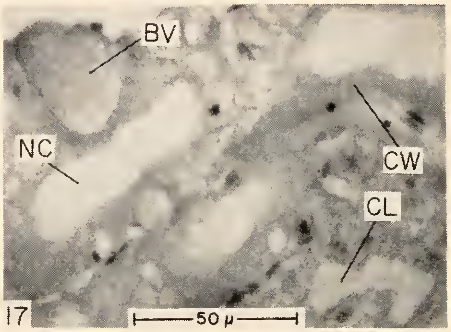
For comparison, Figure 13 also includes data based on observations of the nephridia of *Nereis vexillosa* from the Berkeley Yacht Harbor, San Francisco Bay (Jones, 1957). Although the salinity of the environment at the time of collection was not determined, the means ±2 S.E. for specimens V_1, V_2, and V_4 are clustered around an estimated salinity range, *i.e.*, 73–90% sea water (Jones, 1957, p. 407). The nephridial canals of *N. vexillosa* are significantly larger than those of the down-river forms of *N. limnicola* and are of comparable size to half of the up-river forms.

A comparison of the data of Table II indicates that, in the case of down-river forms, there is no difference between canal diameters of nephridia from the same segment (specimens S-3 and S-13) or from succeeding segments (specimen SB). In up-river forms, there is a significant difference in canal diameters of nephridia from the same segment in two of five cases (specimens S-2 and 49C), and in the one case of nephridia from succeeding segments (specimen RB). In both of the adapted specimens, S-1 and S-4, there is also a significant difference in the case of nephridia from the same segment.









Lettering as in Figures 1–8.

Figures 14 and 15, up-river form of *N. limnicola,* adapted from 0.55% sea water to 118% sea water; Figures 16 and 17, down-river form of *N. limnicola,* adapted from 81% sea water to 0.80% sea water.

FIGURE 14. Dorsal view of right nephridium and associated nephrostome; specimen S-1.
FIGURE 15. View of nephridial tissue; specimen S-1.
FIGURE 16. Dorsal view of right nephridium and associated nephrostome; specimen S-4.
FIGURE 17. View of nephridial tissue; specimen S-4.

Because of these apparently conflicting observations, that is, the small diameter of the nephridial canals of 49E and some of those of S-4 from up-river, and the large diameter of S-1, it is apparent that some physiological and/or physical mechanism, in addition to nephridial canal diameter, operates to allow *N. limnicola* to survive in dilute media.

That a larger canal diameter is advantageous in coping with lowered salinity is suggested by S-4 which apparently developed a larger nephridial canal as it was acclimatized from 81 to 0.80% sea water. That an environment of higher salinity does not necessarily evoke a comparable diminution of canal diameter is suggested by S-1 which apparently maintained a larger canal diameter in one of the measured nephridia while it was acclimatized from 0.55 to 118% sea water.

It would seem, then, that even though the annual fluctuations of salinity in the down-river area may be far greater than those up-river, nephridia with a relatively small diameter are adequate to the osmotic stresses placed on the worms in this area. On the other hand, the nephridia of the down-river forms appear to be more plastic in their response to a fresh-water or near-fresh-water medium; quite possibly, nephridial activity, insofar as water excretion is concerned, may be augmented or superseded by some other mechanism. Finally, there appears to be a general trend toward nephridia of large lumen diameter in the up-river forms, although this is not invariably the case.

I would here extend thanks to Drs. Ralph I. Smith, Kenneth B. DeOme, and Howard A. Bern, all of the Department of Zoology, University of California (where much of the basic work of this study was carried out), to Dr. Larry C. Oglesby of Reed College, and to Drs. Marian H. Pettibone and Clyde Roper, Smithsonian Institution; the advice, criticisms, and suggestions of all of these have been gratefully received, if not always followed. I am particularly obliged to Dr. Smith for the use of a number of specimens of *N. limnicola* from his collections, both sectioned and un-sectioned.

SUMMARY

1. The morphology of the nephridia of specimens of the polychaete worm, *Nereis limnicola* Johnson from areas of different salinity in the estuary of the Salinas River is described.

2. Generally, the canal diameters of the nephridia of the up-river (low salinity) forms are larger than those from down-river (high salinity); the nephridia of the up-river forms are more highly vascularized than those from animals found in higher salinities. This suggests that the nephridial canal acts to rid the animal of the excess water brought into its body by osmotic influx.

3. Nephridial canal diameters of worms adapted from low to high and from high to low salinities approach those of the animals from low salinity; this suggests that a larger canal diameter is efficacious in coping with the osmoregulatory problems presented by a dilute medium, and that canal diameter is not very important in higher salinities.

4. Inconsistencies in the correlation of large nephridial canal diameter with low salinity suggest that other mechanisms are utilized in meeting the stresses imposed by an environment of low salinity.

5. Krishnamoorthi's Index of Excretory Capacity is derived for a number of nephridia; the results indicate that the Index and/or the nephridia of *N. limnicola* do not seem to be comparable with Krishnamoorthi's observations on polychaetes of India.

LITERATURE CITED

AIYAR, R. G., 1933. On the anatomy of *Marphysa gravelyi* Southern. *Rec. Indian Mus., Calcutta*, **35**: 287–323.

BENHAM, W. B., 1891. Nephridium of *Lumbricus* and its blood supply with remarks on the nephridium of chaetopods. *Quart. J. Micr. Sci.*, **32**: 293–334.

BERKELEY, E., AND C. BERKELEY, 1956. A new species and two new records of Polychaeta from eastern Canada. *Canadian J. Zool.*, **34**: 267–271.

DAY, J. H., 1951. The ecology of South African estuaries. Part I. A review of estuarine conditions in general. *Trans. Roy. Soc. South Africa*, **33**: 53–91.

EHLERS, E., 1864–68. Die Borstenwürmer (Annelida Chaetopoda) nach systematischen und anatomischen Untersuchungen dargestellt. Leipzig, W. Engelmann, 748 pp.

FAGE, L., 1906. Recherches sur les organes segmentaires des Annélides Polychètes. *Ann. Sci. Nat. Zool. Paris, Sér. 9*, **3**: 261–410.

GOODRICH, E. S., 1893. On a new organ in the Lycoridea, and on the nephridium in *Nereis diversicolor* O. F. Müll. *Quart. J. Micr. Sci.*, **34**: 387–402.

GOODRICH, E. S., 1895. On the coelom, genital ducts and nephridia. *Quart. J. Micr. Sci.*, **37**: 477–510.

Goodrich, E. S., 1897. On the nephridia of Polychaeta. Part I. On *Hesione, Tyrrhena* and *Nephthys. Quart. J. Micr. Sci.,* **40**: 185–196.

Goodrich, E. S., 1898. On the nephridia of Polychaeta. Part II. *Glycera* and *Goniada. Quart. J. Micr. Sci.,* **41**: 439–457.

Goodrich, E. S., 1900. On the nephridia of the Polychaeta. Part III. The Phyllodocidae, Syllidae, Amphinomidae, etc., with summary and conclusions. *Quart. J. Micr. Sci.,* **43**: 699–748.

Goodrich, E. S., 1945. The study of nephridia and genital ducts since 1895. *Quart. J. Micr. Sci.,* **86**: 113–392.

Hartman, O., 1938. Brackish and fresh-water Nereidae from the northwest Pacific, with the description of a new species from central California. *Univ. California Publ. Zool.,* **43**: 79–82.

Hartman, O., 1944. Polychaetous annelids from California including the description of two new genera and nine new species. *Allan Hancock Pacif. Expeds.,* **10**: 239–307.

Hartman, O., 1960. An account of the nereid worm, *Neanthes diversicolor,* comb. n. in the Caspian Sea and its more extensive distribution. *Zool. Zhurn.,* **39**: 35–39 (in Russian, English summary).

Imajima, M., and O. Hartman, 1964. The polychaetous annelids of Japan. *Allan Hancock Found. Publ., Occas. Papers,* No. 26, pp. 1–452.

Izuka, A., 1908. On the breeding habit and development of *Nereis japonica* n. sp. *Annot. Zool. Japan,* **6**: 295–305.

Johnson, H. P., 1903. Fresh-water nereids from the Pacific Coast and Hawaii, with remarks on fresh-water Polychaeta in general. *Mark Anniv. Vol.,* Art. 10, pp. 205–223.

Jones, M. L., 1957. On the morphology of the nephridium of *Nereis vexillosa* Grube. *Biol. Bull.,* **113**: 407–413.

Khlebovich, V. V., 1963. On the systematic position of the nereid from the Caspian Sea. *Zool. Zhurn.,* **42**: 129–131 (in Russian, English summary).

Krishnamoorthi, B., 1963a. On the distribution of six species of polychaetes in the Adyar Estuary, Madras. *J. Mar. Biol. Assoc. India,* **5**: 97–102.

Krishnamoorthi, B., 1963b. Gross morphology and histology of nephridia in four species of polychaetes. *Proc. Indian Acad. Sci., B,* **57**: 195–209.

Krishnamoorthi, B., 1963c. Volume regulation in eggs, larvae and adults of a brackish-water polychaete, *Diopatra variabilis* (Southern). *Proc. Indian Acad. Sci., B,* **57**: 275–289.

Krishnan, G., 1952. On the nephridia of Nereidae in relation to habitat. *Proc. Natl. Inst. Sci. India,* **18**: 241–255.

Oglesby, L. C., 1965a. Steady-state parameters of water and chloride regulation in estuarine nereid polychaetes. *Comp. Biochem. Physiol.,* **14**: 621–640.

Oglesby, L. C., 1965b. Water and chloride fluxes in estuarine nereid polychaetes. *Comp. Biochem. Physiol.,* **16**: 437–455.

Pettibone, M. H., 1963. Marine polychaete worms of the New England region. 1. Families Aphroditidae through Trochochaetidae. *U. S. Natl. Mus. Bull.,* **227**: 1–356.

Smith, R. I., 1950. Embryonic development in the viviparous nereid polychaete *Neanthes lighti* Hartman. *J. Morph.,* **87**: 417–455.

Smith, R. I., 1953. The distribution of the polychaete *Neanthes lighti* in the Salinas River Estuary, California, in relation to salinity, 1948–1952. *Biol. Bull.,* **105**: 335–347.

Smith, R. I., 1957. A note on the tolerance of low salinities by nereid polychaetes and its relation to temperature and reproductive habit. *L'Année Biol.,* **33**: 93–107.

Smith, R. I., 1958. On reproductive pattern as a specific characteristic among nereid polychaetes. *Syst. Zool.,* **7**: 60–73.

Smith, R. I., 1959a. Physiological and ecological problems of brackish waters. Proc. Twentieth Annual Biol. Colloq., Oregon State College, pp. 59–69.

Smith, R. I., 1959b. The synonymy of the viviparous polychaete *Neanthes lighti* Hartman (1938) with *Nereis limnicola* Johnson (1903). *Pacif. Sci.,* **13**: 349–350.

Smith, R. I., 1963. A comparison of salt loss rate in three species of brackish-water nereid polychaetes. *Biol. Bull.,* **125**: 332–343.

Smith, R. I., 1964. D_2O uptake rate in two brackish-water nereid polychaetes. *Biol. Bull.,* **126**: 142–149.

Stephens, G. C., 1964. Uptake of organic material by aquatic invertebrates. III. Uptake of glycine by brackish-water annelids. *Biol. Bull.,* **126**: 150–162.

CARDIOREGULATION IN LIMULUS. II. GAMMA AMINOBUTYRIC ACID, ANTAGONISTS AND INHIBITOR NERVES

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The neurogenic beat of the *Limulus* heart has long been regarded the classic example of a neurogenic rhythm (Carlson, 1909). As in other neurogenic hearts, such as those of Crustacea, the rate and strength of beating can be decreased by stimulation of cardioinhibitory nerves arising from the central nervous system (Carlson, 1905; Heinbecker, 1933; Pax and Sanborn, 1964). In *Limulus* the decrease in heart rate is not tightly coupled to stimulation of the inhibitor nerves, a time lag in the response occurring both at the beginning and at the end of the stimulation periods. It is probable, therefore, that inhibition in the *Limulus* heart is chemically mediated (Pax and Sanborn, 1964).

The nature of the chemical mediator of inhibition is not known. 5-Hydroxytryptamine (5-HT, serotonin) has been reported to slow the rate of rhythmic discharge from the isolated cardiac ganglion (Burgen and Kuffler, 1957). However, in other neurogenic hearts, 5-HT and related compounds have excitatory effects (Kerkut and Price, 1964).

Gamma-aminobutyric acid (GABA) has also been reported to inhibit the *Limulus* heart (Burgen and Kuffler, 1957). This compound has inhibitory effects on neuromuscular phenomena in a wide variety of other animals. It is present in lobster inhibitory motor neurons but not in excitatory motor neurons (Kravitz *et al.*, 1963). At the crustacean neuromuscular junction, it mimics the action of the inhibitory transmitter both postsynaptically and presynaptically (Dudel, 1965; Takeuchi and Takeuchi, 1966) and in the crustacean cardiac ganglion GABA closely mimics the action of the inhibitor (Florey, 1957; Maynard, 1961). From this evidence it appears possible that GABA or a GABA-like compound may be responsible for cardioinhibition in the *Limulus* heart. We report here results of experiments exploring this possibility more fully.

A primary requirement of any supposed transmitter is that, when artificially applied, it mimics in all respects stimulation of the prejunctional structure (McLennan, 1963). Stimulation of the cardioinhibitory nerves in *Limulus* results in a decrease in rate and strength of beating of the intact heart, a decrease in the number of units discharging in the cardiac ganglion during each burst of electrical activity and a decrease in the total duration of each burst (Carlson, 1905; Heinbecker, 1933; Pax and Sanborn, 1964). We have tested the ability of exogenously applied GABA to mimic these actions of the inhibitor nerves.

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Since data obtained by application of supposed transmitters to the cardiac ganglion are at best equivocal, we have also followed a second line of investigation. Compounds which block the action of the endogenous transmitter should similarly antagonize the effects of exogenously applied GABA.
this purpose we have used picrotoxin, a compound capable of blocking the action of GABA in other systems (Van der Kloot *et al.*, 1958). We have tested picrotoxin for its ability to block the action of the endogenous transmitter, *i.e.*, block the action of the inhibitor nerves. We have also tested picrotoxin for its ability to block the action of the supposed transmitter artificially applied to the heart. For

MATERIALS AND METHODS

Source and maintenance of animals

Adult *Limulus polyphemus*, maintained as previously described (Pax and Sanborn, 1964), 20 to 25 cm. maximal width, were used in all experiments. They were shipped by air express at two-week intervals from the Supply Department, Marine Biological Laboratory, Woods Hole, Massachusetts, and maintained in moist excelsior at a temperature of 5° C. Responses of animals so maintained did not vary for at least six weeks.

Animal preparations

Isolation of the heart from *Limulus* requires removal of the tough dorsal exoskeleton. This is best done by sawing through the exoskeleton just lateral to the underlying heart and joining the lateral cuts with transverse anterior and posterior cuts so that a rectangular piece of isolated exoskeleton overlying the heart may be removed by lifting and scraping it free of the underlying tissues. Once this piece of exoskeleton has been removed the internal extensor muscles of the opisthosoma dorsal to the heart in the cephalothorax and the epidermal tissue overlying it in the opisthosoma can be dissected away. The intact heart can then be removed.

Stimulation of inhibitor nerves

As we suggested earlier (Pax and Sanborn, 1964), stimulation of the inhibitor nerves near the ventral nerve ring is undesirable since they also contain fibers which innervate muscles. We have since been able to locate inhibitor fibers as they enter the heart dorsally. At these sites the nerves apparently consist exclusively of cardioinhibitory fibers.

Perfusion of the isolated heart

After removal from the animal the heart was placed in a V-shaped lucite chamber 15 cm. in length. The heart was ligated anteriorly in the second segment and posteriorly a cannula was inserted into the lumen of the heart through the cardiac muscle.

Tension on the heart walls and the amount of intra-lumenal pressure both influence the rate and the strength of beating of the heart (Carlson, 1907). Longitudinal tension approximating that on the heart *in situ* was obtained by stretching the heart to a length equal to that present before removal from the animal.

In order to maintain an intra-lumenal pressure, a gravity-feed reservoir of Chao's (1933) saline solution (0.44 *M* NaCl, 0.009 *M* KCl, 0.037 *M* $CaCl_2$) was connected to the cannula at the posterior of the heart. The hearts were perfused at the rate of 20 ml. per minute, the route of the perfusion fluid being from the lumen of the heart out through the ostia and lateral arteries to the exterior. The total volume of fluid in the chamber was maintained at 10 ml. by providing an overflow in the chamber near the anterior end of the heart.

Recording of data

Electrical activity was recorded from the cardiac ganglion of the intact heart by dissecting it free of the heart muscle in the second and the third segments and placing it over hooked platinum electrodes. From the isolated cardiac ganglion, electrical activity was recorded by stringing the ganglion through a series of 12 platinum loop electrodes spaced five mm. apart. During the course of a single experiment any of these electrodes could be chosen to be used as recording electrode. Measurement of mechanical activity of the heart muscle was obtained with a Statham G10b displacement transducer (maximum displacement 0.15 oz.).

Experimental methods and drugs

All drugs were dissolved in Chao's (1933) saline as shortly before use as practicable. Parallel reservoirs of saline and drug solution were connected to the chamber through a two-channel stopcock so that perfusion could be alternated by a turn of the barrel.

Data reduction

Heart rates in *Limulus* vary greatly from animal to animal (Pax and Sanborn, 1964). Moreover those hearts which have an initial high rate of beating tend to have a greater change in rate during inhibition than do those which have an initial low rate of beating. For these reasons we have, when measuring changes in rate, used each animal as its own control and expressed all rates as relative heart rates. Relative heart rate is defined as the ratio of the experimentally altered rate to the control rate. Thus relative rates of less than one are indicative of inhibition and values greater than one indicate excitation. In a similar manner, all data on strength of contraction of the heart muscle are expressed as relative strengths.

In drug perfusion experiments relative rates and contraction strengths were calculated from the mean rates and contraction strengths during the last two minutes of perfusion. In stimulation experiments relative rates were calculated from the mean rates during the entire time of stimulation.

RESULTS

Gamma-aminobutyric acid

Perfusion of GABA through the intact isolated *Limulus* heart results in a decreased heart rate. A typical result of such perfusion is shown in Figure 1, and the results of 21 such perfusions in 15 different hearts are plotted in Figure 2. The solid line in the figure is the regression line for these data as determined by

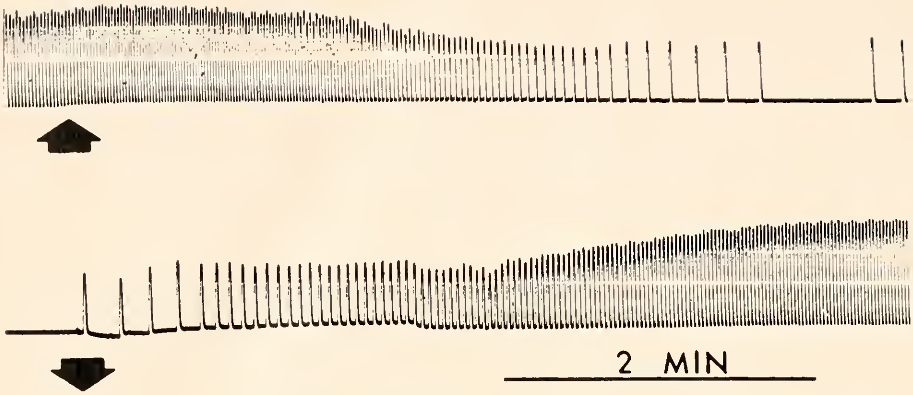

FIGURE 1. The response of the isolated heart to perfusion with GABA. The record is continuous from upper left to lower right. One hundred ml. of 5×10^{-5} *M* GABA were perfused during the time between the two arrows.

the method of least squares; the standard error is indicated by the dashed lines. The slope of the regression line is − 0.36, the standard error 0.23. At all concentrations of GABA tested, the rate-slowing effect is readily reversible by perfusion with drug-free saline (Fig. 1).

The strength of contraction of the heart also decreases with GABA perfusion (Fig. 1). In Figure 3 the relationship between relative strength and concentration

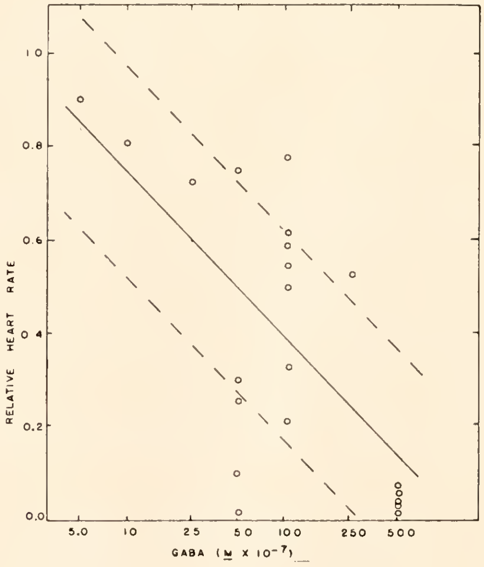

FIGURE 2. Relation of relative heart rate to concentration of perfused GABA. Each point represents a single perfusion. The solid line is the regression line determined by least squares and the dashed lines are the standard error of the regression line.

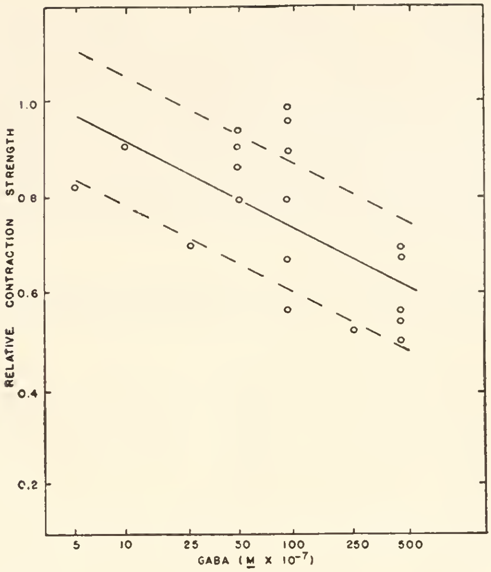

FIGURE 3. Relation of relative contraction strength to concentration of GABA perfused through isolated hearts. The solid line is the regression line determined by least squares and the dashed lines represent the standard error of the regression line.

of perfused GABA is plotted for 19 perfusions in 13 different hearts. The slope of the regression line in this case is only − 0.17 compared to the slope of − 0.36 for rate changes. Thus a concentration of GABA sufficient to reduce heart rate by 50% reduces contraction strength by less than 20%.

Although GABA reduces the rate at which rhythmic bursts of electrical activity occur in isolated cardiac ganglia, it causes no readily apparent changes in the pattern of the individual bursts. In Figure 4 the pattern of a typical burst of electrical

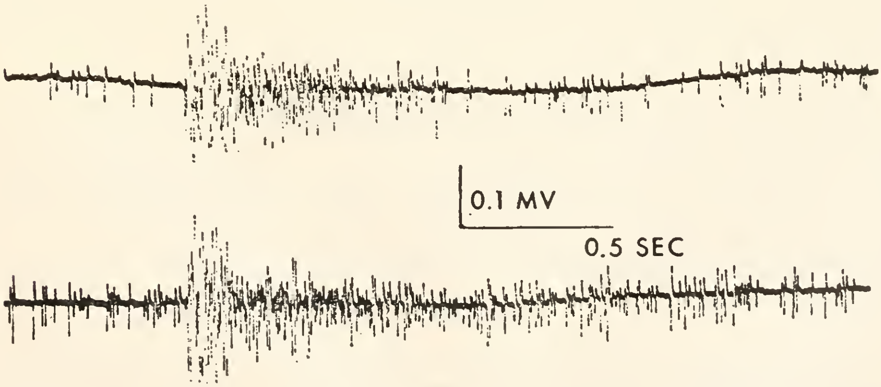

FIGURE 4. The pattern of electrical activity in an isolated ganglion. The upper trace shows a representative burst before drug treatment; the lower trace a representative burst after bathing the ganglion for one minute in 1×10^{-5} *M* GABA.

activity recorded from the fourth segment of an isolated cardiac ganglion in drug-free saline is compared to a typical burst of electrical activity recorded from the same segment of the same isolated cardiac ganglion after bathing in 1×10^{-5} *M* GABA for one minute. At the time of recording the rate of rhythmic bursting has been reduced by 50% but, contrary to the changes seen in the pattern of the burst during stimulation of the inhibitor nerves, there is neither a decrease in the duration of the burst nor a lesser number of discharges in a particular burst. Bathing the ganglion for one minute in drug-free saline is sufficient to return the rate of rhythmic bursting to the pre-treatment level.

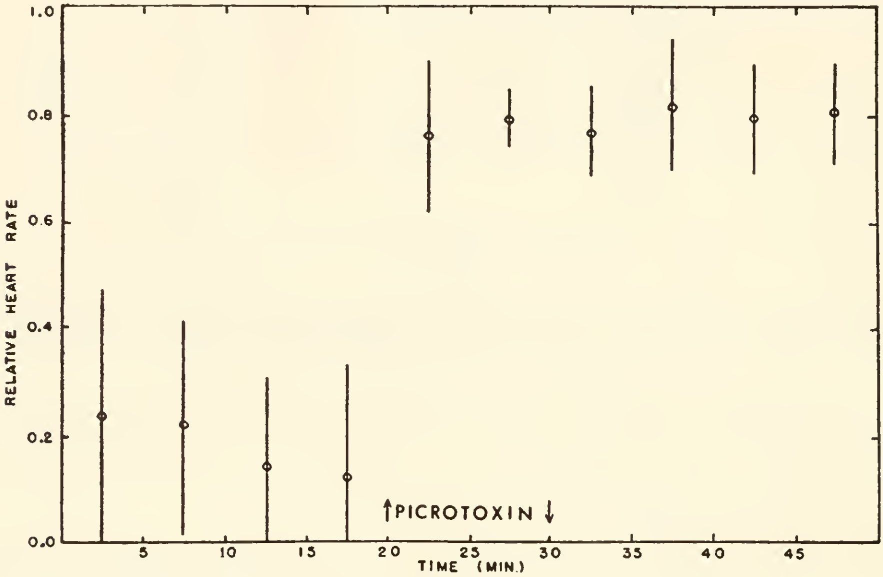

FIGURE 5. Effect of picrotoxin on cardioinhibitory nerves. Each point represents the mean relative heart rate for four hearts during stimulation of the inhibitor nerves. Vertical lines extend one standard deviation on either side of the mean. During the time between the two arrows 100 ml. of 10^{-3} *M* picrotoxin were perfused.

L-glutamic acid at a concentration of 10^{-3} *M* perfused through the isolated heart reduces the strength of contraction of the heart muscle to a barely detectable level but does not change the heart rate. At 10^{-5} *M* it has no measurable effects on rate or strength. In like manner carnitine (gamma-aminobutyric-beta-hydroxy-betaine) perfused through the heart at 10^{-4} *M* causes a marked decrease in strength of contraction but causes no measurable change in rate.

Picrotoxin

The ability of picrotoxin to block the action of the inhibitor nerves was tested in four isolated hearts. In each experiment the inhibitor nerve was stimulated near its junction with the cardiac ganglion in the fourth heart segment. Stimulation was given for 40 seconds out of every five minutes. During the first four such five-minute intervals drug-free saline was perfused. In the fifth and sixth intervals 100 ml. of 10^{-3} *M* picrotoxin were perfused and then during the next four five-minute intervals drug-free saline was again perfused.

In each case picrotoxin alone caused an increase in heart rate, the mean rate being 24.9 beats per minute before picrotoxin perfusion and 34.6 beats per minute after picrotoxin perfusion. To compensate for this drug-induced rate increase, the relative rates in the portion of the experiment when picrotoxin was used were computed by comparing the ratio of the rate *during* stimulation to that obtained immediately *before* stimulation. Both rates were thus measured in the presence of the drug.

In each of the four hearts the inhibitor nerves were less effective during picrotoxin perfusion. In two of these this decreased effectiveness preceded the increase in heart rate. The block of the inhibitor nerves, therefore, is not merely a reflection of the increased heart rate caused by the picrotoxin. The mean relative rate obtained by stimulation before treatment with the drug was 0.19 (SD = 0.15) *i.e.*, stimulation caused an 81% decrease in rate, while after picrotoxin treatment the mean relative rate was 0.76 (SD = 0.10). Thus, in the presence of the drug, stimulation decreased the rate by only 24%. A "*t*" test for the difference between these two means showed it to be significant ($P > 0.99$). In Figure 5 the mean relative rate produced by stimulation of the inhibitor nerves in the four hearts before, during and after perfusion with picrotoxin during each of the ten stimulation periods is shown. The mean decrease in rate produced by stimulation of the inhibitor nerves in the four hearts before picrotoxin perfusion was 20.2 beats per minute. During and after picrotoxin perfusion the decrease was 8.3 beats per

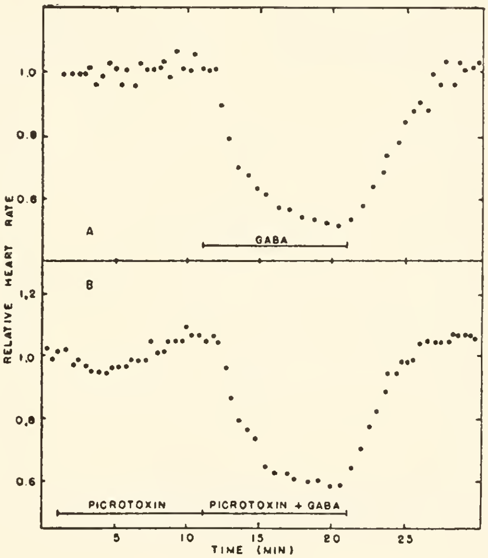

FIGURE 6. Rate changes in a heart perfused with GABA alone (2×10^{-5} *M*) and with GABA plus picrotoxin (1×10^{-3} *M*). See text for details.

minute. We have no data concerning changes in contraction strength during stimulation of inhibitor nerves while perfusing with picrotoxin.

The reduced effectiveness of the inhibitor nerves outlasts the perfusion with picrotoxin. As can be seen from Figure 5 the mean relative rate obtained by stimulation 20 minutes after the end of perfusion with picrotoxin was still 0.80 (SD = 0.09), 0.60 unit greater than the mean relative rate obtained before picrotoxin treatment.

Since picrotoxin is effective in blocking the function of the cardioinhibitory nerves of *Limulus,* its ability to antagonize the action of applied GABA was also tested. Four isolated hearts were used in these experiments. Since from one preparation to the next there is considerable variation in the response to a given concentration of GABA, a control perfusion of 100 ml. of GABA was made for each heart prior to picrotoxin treatment. After the heart had recovered from the GABA perfusion by perfusing for one-half hour with drug-free saline, treatment with picrotoxin was begun. After perfusion with 100 ml. of 10^{-3} *M* picrotoxin in saline, a second 100-ml. portion containing the same concentration of GABA as that previously given was perfused. In this way GABA at concentrations of 5, 10 and 20×10^{-6} *M* was tested against picrotoxin at 10^{-3} *M*.

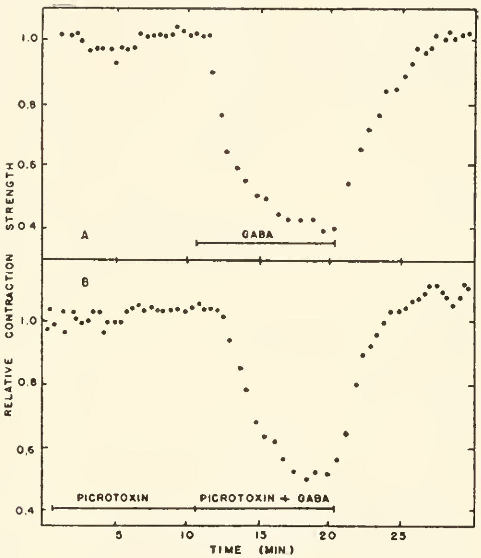

FIGURE 7. Contraction strength changes in a heart perfused with GABA alone (2×10^{-5} *M*) and with GABA plus picrotoxin (1×10^{-3} *M*). See text for details.

The response of the heart to GABA is not significantly altered by picrotoxin. Figure 6 presents the results for one of the four hearts. In the example shown the mean decrease in rate was 8.7 beats per minute during GABA perfusion prior to picrotoxin treatment. During GABA perfusion after picrotoxin treatment the mean decrease in rate was 7.7 beats per minute. Not only is the decrease in rate almost identical in the presence or absence of picrotoxin, but the time course of the response to GABA is essentially unaltered. Although there were differences between relative rates obtained during GABA perfusion before and after picrotoxin treatment in individual hearts, the mean relative rate for the four hearts during GABA perfusion prior to picrotoxin treatment was 0.30, exactly the same as the mean value obtained during GABA perfusion after picrotoxin treatment (Mean Difference = 0.00; SD = 0.05).

In one of the hearts in which the interaction between GABA and picrotoxin was tested, data about strength changes were also obtained (Fig. 7). During perfusion with GABA alone the minimal relative contraction strength was 0.43. When GABA and picrotoxin were perfused together it was 0.50. The time course of the inhibition in both cases was approximately the same.

Discussion

We have considered the evidence that GABA acts as a synaptic transmitter in the cardioinhibitory pathway of *Limulus*. It is worthwhile comparing our observations with those on other arthropod systems in which GABA is believed to be a junctional transmitter.

In crustacean inhibitory motor neurons, GABA clearly appears to be the natural transmitter. It duplicates the effects of activation of the inhibitory neurons on muscle (Dudel, 1965; Takeuchi and Takeuchi, 1966), is present in the inhibitory axons and the synthetic machinery is present in such axons (Kravitz *et al.*, 1963).

Nearly as conclusive evidence exists that GABA is the natural transmitter for cardioinhibition in crustaceans. While it has not been isolated from this site, application to the ganglion cells of the crustacean heart has been shown to mimic, in all respects, the action of the natural transmitter (Florey, 1957; Maynard, 1961).

On the other hand, although the crustacean stretch receptor has been shown to be inhibited by GABA it does not appear to be the transmitter in this system (Kuffler and Edwards, 1958; Edwards and Kuffler, 1959).

Unequivocal proof that a given compound is the endogenous transmitter at a given junction is not easily obtained. Short of actual demonstration that the supposed transmitter is liberated by activity in the presynaptic fibers and that it, when applied in physiological concentrations, reproduces the conductance changes which occur during synaptic transmission (Terzuolo and Edwards, 1962), some doubt about the identity of the transmitter will exist. Because of the anatomical arrangement at many junctions it is difficult, if not impossible, to produce such direct evidence about the nature of the transmitter.

In view of this difficulty a number of other sets of criteria have been proposed which do not rely on such direct evidence. One such set is that of McLennan (1963): (1) The substance occurs in presynaptic structures. (2) An enzymatic mechanism for synthesis of the substance is present. (3) An enzyme system for inactivation of the substance is present. (4) Application of the substance mimics stimulation. (5) During stimulation the substance is detectable in perfusates. (6) Pharmacological agents which interfere with operation of the neuron similarly affect the action of the substance artificially applied.

If a given chemical is to be seriously considered to be the endogenous transmitter at a given junction then it must meet each of these criteria. Conversely, if a given chemical does not meet one or more of these criteria, it is doubtful that it is the endogenous transmitter at that junction. Our evidence shows that GABA fails to meet two requirements. First, picrotoxin which effectively blocks the action of the inhibitor nerves is without effect upon the slowing of the heart rate caused by GABA (Criterion 6). Since the exact site of action of picrotoxin at the inhibitory junction is unknown, failure to meet this requirement alone is not sufficient to eliminate GABA as a possible transmitter at this junction. However GABA also fails to meet a second requirement, namely that it mimic stimulation of the inhibitor nerve (Criterion 4). Although stimulation and GABA both slow the heart rate, they have quite different effects upon the pattern of neural activity in the cardiac ganglion. Stimulation of the inhibitor nerves decreases the number of units discharging in the cardiac ganglion during each burst of electrical activity, as well as the total duration of each burst (Heinbecker, 1933). GABA produces neither of these changes in the pattern of the burst.

We believe, therefore, that even if GABA were to meet some of the other criteria listed above, it could not be seriously considered as a natural transmitter in the *Limulus* cardioinhibitory pathway.

The authors wish to express their thanks to the National Science Foundation for support for certain phases of these studies.

SUMMARY

1. GABA (5×10^{-7} to 5×10^{-5} M) perfused through the isolated *Limulus* heart mimics stimulation of the cardioinhibitory nerves by decreasing rate and strength of beating of the heart.

2. GABA, unlike activity in the cardioinhibitory nerves, decreases neither the number of units discharging nor the total duration of each burst of electrical activity in the cardiac ganglion.

3. Picrotoxin (1×10^{-3} M) blocks the function of the cardioinhibitory nerves.

4. Picrotoxin (1×10^{-3} M) blocks neither the rate nor the strength-decreasing effects of applied GABA.

5. Since GABA does not mimic the action of the inhibitor nerves and its action is not blocked by an agent blocking the function of the inhibitor nerves, we believe it is probable that GABA is not a transmitter in the *Limulus* cardioinhibitory pathway.

LITERATURE CITED

BURGEN, A. S. V., AND S. W. KUFFLER, 1957. Inhibition of the cardiac ganglion of *Limulus polyphemus* by 5-hydroxytryptamine. *Biol. Bull.*, **113**: 336.

CARLSON, A. J., 1905. The nature of cardiac inhibition with special reference to the heart of *Limulus*. *Amer. J. Physiol.*, **13**: 217–240.

CARLSON, A. J., 1907. On the mechanism of the stimulating action of tension on the heart. *Amer. J. Physiol.*, **18**: 149–155.

CARLSON, A. J., 1909. Vergleichende Physiologie der Herznerven und der Herzganglien bei den Wirbellosen. *Ergebn. Physiol.*, **8**: 371–462.

CHAO, I., 1933. Action of electrolytes on the dorsal median nerve cord of the *Limulus* heart. *Biol. Bull.*, **64**: 358–382.

DUDEL, J., 1965. Presynaptic and postsynaptic effects of inhibitory drugs on crayfish neuromuscular junction. *Pflügers Archiv.*, **283**: 104–118.

Edwards, E., and S. W. Kuffler, 1959. The blocking effect of γ-aminobutyric acid (GABA) and the action of related compounds on single nerve cells. *J. Neurochem.*, **4**: 19–30.

Florey, E., 1957. Further evidence for the transmitter function of factor I. *Naturwissenschaften*, **44**: 424–425.

Heinbecker, P., 1933. The heart and median cardiac nerve of *Limulus polyphemus*. *Amer. J. Physiol.*, **103**: 104–120.

Kerkut, G. A., and M. A. Price, 1964. Chromatographic separation of cardio-accelerators (6-HT and a mucopeptide) from *Carcinus* heart. *Comp. Biochem. Physiol.*, **11**: 45–52.

Kravitz, E. A., S. W. Kuffler and D. D. Potter, 1963. Gamma aminobutyric acid and other blocking compounds in Crustacea III. Their relative concentrations in separated motor and inhibitory axons. *J. Neurophysiol.*, **26**: 739–751.

Kuffler, S. W., and C. Edwards, 1958. Mechanism of gamma aminobutyric acid (GABA) action and its relation to synaptic inhibition. *J. Neurophysiol.*, **21**: 589–610.

Maynard, D. M., 1961. Cardiac inhibition in a decapod crustacean. *In*: Nervous Inhibition, E. Florey, Ed., Pergamon Press, New York, pp. 148–178.

McLennan, H., 1963. Synaptic Transmission. W. B. Saunders Co., Philadelphia.

Pax, R. A., and R. C. Sanborn, 1964. Cardioregulation in *Limulus*. I. Physiology of inhibitor nerves. *Biol. Bull.*, **126**: 133–141.

Takeuchi, A., and N. Takeuchi, 1966. On the permeability of the presynaptic terminal of the crayfish neuromuscular junction during synaptic inhibition and the action of γ-aminobutyric acid. *J. Physiol.*, **183**: 433–449.

Terzuolo, C. A., and C. Edwards, 1962. Excitation and synaptic transmission. *Ann. Rev. Physiol.*, **24**: 325–356.

Van der Kloot, W. G., J. Robbins and I. M. Cooke, 1958. Blocking by picrotoxin of peripheral inhibition in crayfish. *Science*, **127**: 521–522.

CARDIOREGULATION IN LIMULUS. III. INHIBITION BY 5-HYDROXYTRYPTAMINE AND ANTAGONISM BY BROMLYSERGIC ACID DIETHYLAMIDE AND PICROTOXIN

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Cardioinhibition in *Limulus* appears to be chemically mediated. The decrease in heart rate resulting from stimulation of the inhibitor nerves is not tightly coupled to the stimulation, a time lag in the response occurring both at the beginning and at the end of the stimulation periods (Carlson, 1905; Pax and Sanborn, 1964).

The nature of the chemical mediator of inhibition is not known. A variety of pharmacological agents have been tested since the neurogenic nature of the heart beat was first shown by Carlson (1904). Of these, only three have been reported to cause a decrease in heart rate: ergot (Carlson, 1906), 5-hydroxytryptamine (5-HT) and gamma-aminobutyric acid (GABA) (Burgen and Kuffler, 1957).

A study of GABA as the possible inhibitory neurotransmitter in the *Limulus* heart has previously been reported (Pax and Sanborn, 1967). Although this compound decreases rate and strength of beating when applied artificially, it does not decrease the number of units discharging or the total duration of each burst of electrical activity in the cardiac ganglion as does stimulation of the inhibitor nerves. Moreover, picrotoxin, though effective in blocking the function of the inhibitor nerves, is not an effective antagonist to GABA activity. It appears, therefore, that GABA is not involved as the inhibitory neurotransmitter in the *Limulus* heart.

5-HT, like GABA, is found in a wide variety of animals (Welsh and Moorhead, 1960). It has been shown to have physiological significance in such diverse animal groups as flatworms and vertebrates (Mansour *et al.,* 1960). In contrast to its reported inhibitory action on the *Limulus* heart (Burgen and Kuffler, 1957) it has an excitatory effect on the crustacean neurogenic heart (Florey and Florey, 1954; Maynard and Welsh, 1959; Kerkut and Price, 1964; Cooke, 1966). We report here results of experiments exploring more fully the possibility that 5-HT or a 5-HT-like compound is the cardioinhibitory transmitter in the neurogenic *Limulus* heart.

MATERIALS AND METHODS

Materials and methods are as previously described (Pax and Sanborn, 1967).

RESULTS

5-Hydroxytryptamine

Perfusion of 5-HT through the isolated heart results in a decrease in heart rate. A typical result of 5-HT perfusion is shown in Figure 1. In Figure 2 the

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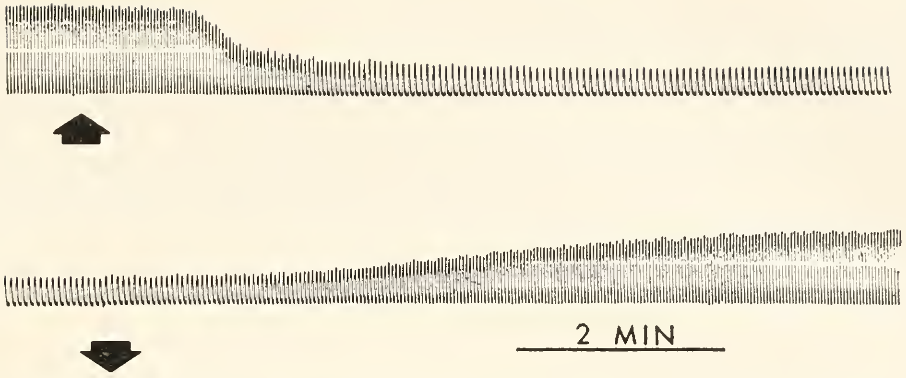

Figure 1. Response of the isolated heart to perfusion of 5-HT. During the time between the arrows 100 ml. of 5 × 10^{-6} *M* 5-HT were perfused through the heart.

relationship between concentration of 5-HT perfused and relative heart rate is plotted for 18 perfusions of 5-HT in 12 different hearts. The solid line on the graph is the regression line for these data as determined by the method of least squares. The standard error of this line is indicated by the dashed lines on either side of the regression line. The slope of this regression line is −0.34; the standard error 0.17. The threshold for rate changes, as determined by solving the equation

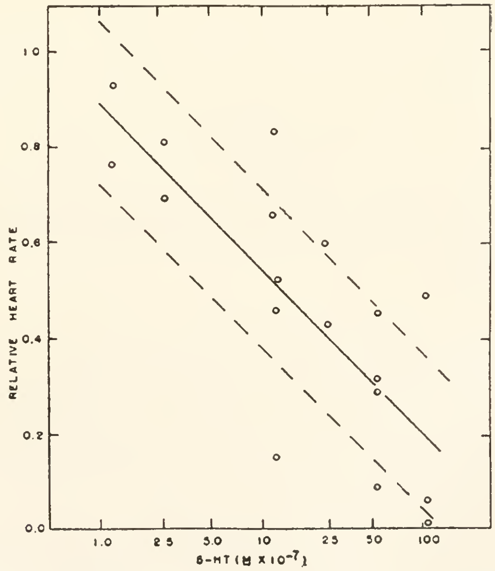

Figure 2. Relation of relative heart rate to concentration of 5-HT perfused through the isolated heart. Each point represents a single perfusion. The solid line is the regression line determined by the method of least squares and the dashed line is the standard error of the regression line.

for the regression line, is 4.9×10^{-8} *M* while at 4.1×10^{-5} *M* 5-HT a relative heart rate of zero would be expected.

The strength of heart beat also decreases when 5-HT is perfused through the isolated heart (Fig. 1). In Figure 3 the relationship between 5-HT concentration and relative contraction strength is plotted for 14 perfusions of 5-HT in nine different hearts. The calculated threshold concentration for strength changes is 5.6×10^{-8} *M*, about the same as that calculated for rate changes, but the calculated regression lines for rate and strength changes are not parallel (slope = −0.34 for rate, −0.31 for strength).

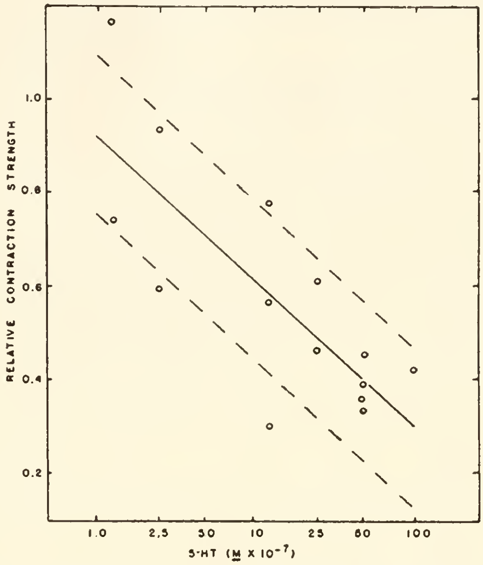

FIGURE 3. Relation of relative contraction strength to concentration of 5-HT perfused through the isolated heart. Each point represents a single perfusion. The solid line is the regression line determined by the method of least squares and the dashed lines are the standard error of the regression line.

Both of the above effects of 5-HT are readily reversible. Perfusion with drug-free saline for five minutes following drug treatment is usually sufficient to bring the rate and strength of beating within 10% of their pre-treatment levels.

Neither 5-hydroxytryptophan—the precursor of 5-HT—nor 5-hydroxyindoleacetic acid—its major metabolite—at 10^{-4} *M* had any detectable effects on rate or strength of beating of the isolated heart.

Electrical activity of the isolated cardiac ganglion is also affected by 5-HT treatment. The rate of rhythmic discharges decreases. The number of units discharging in each burst is reduced and the total duration of each burst is lessened. The pattern of a typical burst of electrical activity recorded from the fourth segment of the isolated cardiac ganglion before treatment and after treatment with 1×10^{-6} *M* 5-HT is shown in Figure 4. In this experiment the relative heart rate during perfusion of 5-HT was 0.51. The changes in the pattern of electrical activity in the ganglion during a particular burst are readily apparent.

After treatment of isolated ganglia with 5-HT, one minute of bathing in drug-free saline returns the rate to the pre-treatment level.

Bromlysergic acid diethylamide

Bromlysergic acid diethylamide (BOL) is a potent and specific antagonist of 5-HT in other animals (Gyermek, 1961). Since 5-HT appears to mimic the action of the inhibitor nerves of the *Limulus* heart we have studied the interaction between BOL and the inhibitor nerves. The function of the inhibitor nerves was tested in four animals before, during, and after perfusion with 1.6×10^{-5} *M* BOL. These experiments were performed in a manner parallel to that used in testing the interaction of the inhibitor nerves and picrotoxin (Pax and Sanborn, 1967). Nerves were stimulated for 20 seconds out of every five minutes. During the first five five-minute stimulation intervals, drug-free saline was perfused. During the next two five-minute intervals 100 ml. of BOL were perfused and during the last six five-minute intervals drug-free saline was again perfused.

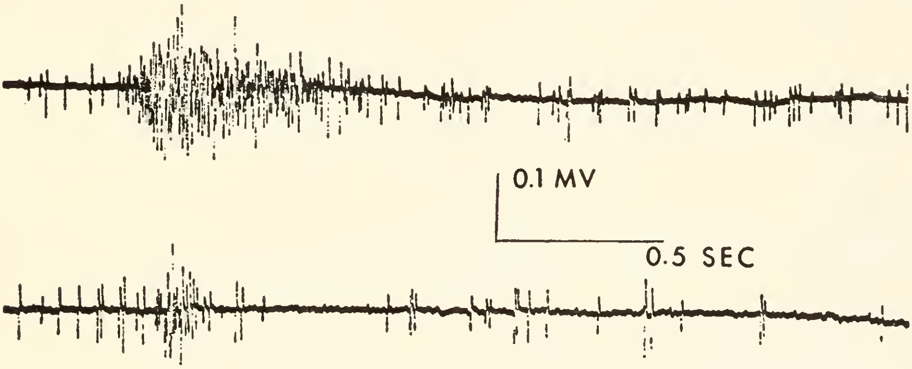

FIGURE 4. Changes in the pattern of electrical activity in the isolated ganglion resulting from 5-HT treatment. The upper trace is a representative burst before drug treatment, the lower trace a representative burst after bathing the ganglion for one minute in 1×10^{-6} *M* 5-HT.

BOL (1.6×10^{-5} *M*) alone causes a slight increase in heart rate, the mean rate for 15 different hearts being 30.3 beats per minute before BOL treatment and 31.8 beats per minute after BOL treatment. The relative rate for each of the 13 stimulation periods was computed by taking the ratio of the rate during the stimulation period to the rate just previous to that same stimulation period.

BOL is an effective antagonist of inhibitor nerve action in the *Limulus* heart. The mean relative rate during stimulation of the inhibitor nerves for each of the 13 stimulation periods is shown in Figure 5. Stimulation of the inhibitor nerves before BOL treatment resulted in a mean decrease in rate of 19.8 beats per minute. The relative rate was 0.28 (SD = 0.11), *i.e.,* stimulation reduced the rate by 72%. After BOL treatment the decrease in rate was 12.0 beats per minute and the relative rate was 0.66 (SD = 0.12), *i.e.,* stimulation reduced the rate by only 34%.

A "t" test for the difference between the two relative rates showed the inhibitor nerves significantly less effective in decreasing heart rate after BOL treatment ($P > 0.95$). Function of the inhibitor nerves does not begin to return to the pre-BOL perfusion level even after perfusion with drug-free saline for as long as 30 minutes (Fig. 5).

Since BOL blocks the function of the cardioinhibitory nerves in *Limulus,* it should also antagonize the action of artificially applied 5-HT, if 5-HT is acting at a junction in the cardioinhibitory pathway. The ability of BOL to antagonize the action of 5-HT was tested on four isolated hearts.

These experiments were performed in a manner parallel to our experiments testing the interaction of GABA and picrotoxin. One hundred ml. of saline containing 5-HT were initially perfused through each heart to calibrate its response. After one-half hour of perfusion with drug-free saline to eliminate the effects of the 5-HT, 100 ml. of 1.6×10^{-5} M BOL were perfused. This perfusion was immediately followed by perfusion of 100 ml. of 1.6×10^{-5} M BOL to which had been added the same concentration of 5-HT as that previously given during the calibration perfusion. 5-HT at concentrations of 1 and 5×10^{-6} M was tested in this way against BOL at 1.6×10^{-5} M.

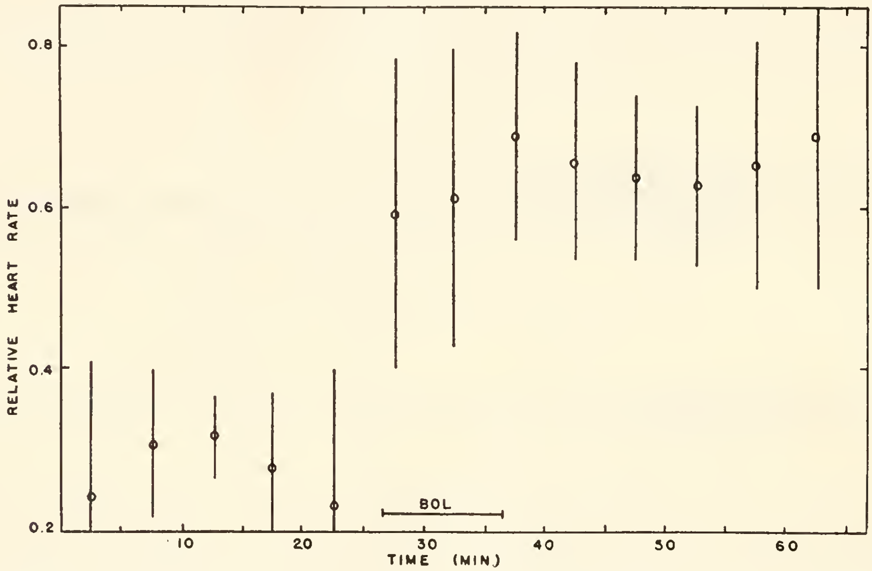

FIGURE 5. Effect of BOL on the function of the cardioinhibitory nerves. Each point represents the mean relative heart rate for four hearts during stimulation of the inhibitor nerves. Vertical lines extend one standard deviation on either side of the mean. During the time between the two arrows 100 ml. of 1.6×10^{-5} M BOL were perfused.

Our experiments show that this concentration of BOL is an effective antagonist of the rate-decreasing effects of 5-HT. The mean relative rate with 5-HT perfusion prior to BOL treatment was 0.57 (mean decrease in rate, 12.9 beats per minute) while after BOL treatment it was 0.93 (mean decrease in rate, 2.1 beats per

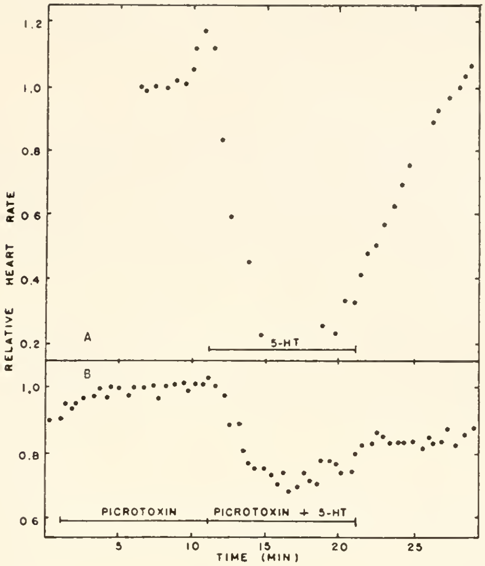

FIGURE 6. Rate changes in a heart perfused with 5×10^{-6} M 5-HT alone and with 5-HT plus 1.6×10^{-5} M BOL. See text for details.

minute). A "t" test for the difference between the two relative rates showed 5-HT significantly less effective in reducing heart rate after BOL treatment ($P > 0.99$). The results of a typical experiment are shown in Figure 6.

BOL (1.6×10^{-5} M) is also an effective antagonist of the strength-decreasing effects of artificially applied 5-HT. The mean relative strength with 5-HT perfusion prior to BOL treatment was 0.41 while after BOL treatment it was 1.04. A

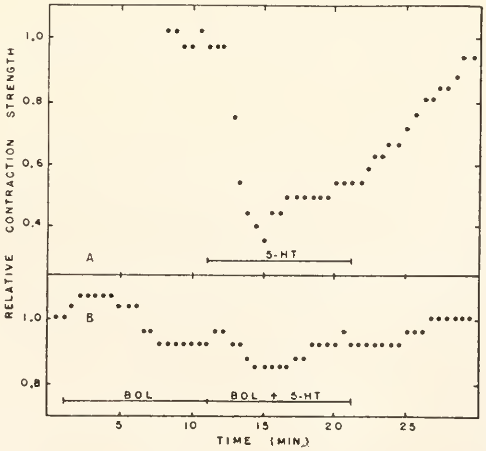

FIGURE 7. Changes in contraction strength with perfusion of 5×10^{-8} M 5-HT alone and with 5-HT plus 1.6×10^{-5} M BOL. See text for details.

"*t*" test for the difference between these means showed it significant ($P > 0.99$). Results of a typical experiment are shown in Figure 7.

Picrotoxin

Picrotoxin has previously been shown to be effective in blocking the action of the inhibitor nerves in *Limulus* (Pax and Sanborn, 1967). Since it blocks the inhibitor nerves it should also antagonize the action of applied 5-HT, if 5-HT is acting as a neurotransmitter in the cardioinhibitory pathway. We have therefore tested the ability of picrotoxin to block the action of applied 5-HT on four isolated hearts.

The experiments were performed in a manner parallel to that described for testing the interaction of 5-HT and BOL. One hundred ml. of saline containing 5-HT were initially perfused through each heart to determine a control response. After one-half hour of perfusion with drug-free saline to eliminate the effects of the 5-HT, 100 ml. of 10^{-3} *M* picrotoxin were perfused followed immediately by 100 ml. of 10^{-3} *M* picrotoxin to which had been added the same concentration of 5-HT as that given during the control perfusion. 5-HT at concentrations of 1, 5, and 10×10^{-6} *M* was tested in this way against picrotoxin at 10^{-3} *M*.

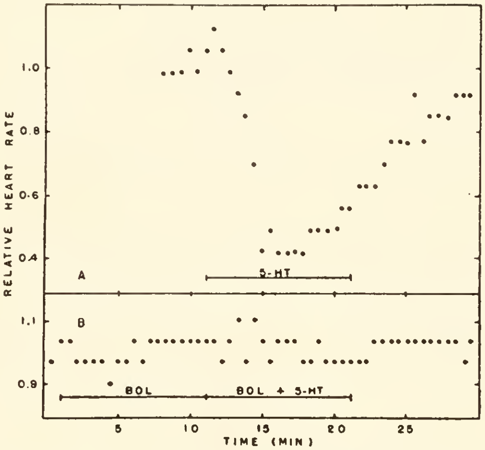

FIGURE 8. Rate changes in a heart perfused with 5×10^{-6} *M* 5-HT alone and with 5-HT plus 1×10^{-3} *M* picrotoxin. See text for details.

As with BOL, there is antagonism between 5-HT and picrotoxin. The mean relative rate with 5-HT perfusion prior to picrotoxin treatment was 0.34 (mean decrease in rate, 18.2 beats per minute) while after picrotoxin treatment it was 0.75 (mean decrease in rate, 11.5 beats per minute). A "*t*" test for the difference between the two relative rates showed the relative rate to be significantly higher after picrotoxin treatment ($P > 0.99$). The results of a typical experiment are presented in Figure 8.

By contrast, the effects upon relative contraction strength are quite different. In this variable, picrotoxin and 5-HT show synergism rather than antagonism.

Though no measurable change in contraction strength is brought about by perfusion of picrotoxin alone, when picrotoxin is perfused with 5-HT a greater decrease in contraction strength occurs than when 5-HT alone is perfused. The mean relative strength of four hearts with 5-HT perfusion prior to picrotoxin treatment was 0.51 while after picrotoxin treatment it was 0.26. A "*t*" test for the difference between these means showed the relative contraction strength significantly lower after picrotoxin treatment than before ($P > 0.95$). The results of a typical experiment are presented in Figure 9.

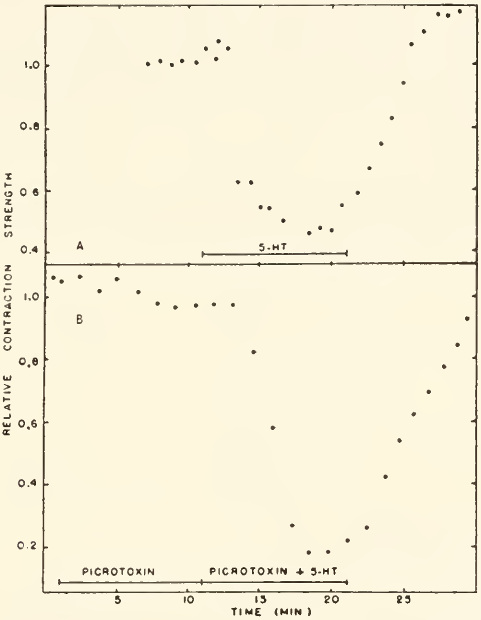

FIGURE 9. Changes in contraction strength with perfusion of 5×10^{-6} *M* 5-HT alone and with 5-HT plus 1×10^{-3} *M* picrotoxin. See text for details.

BOL and GABA

BOL blocks the function of the cardioinhibitory nerves. GABA, although it decreases the rate and strength of beating of the intact heart, does not alter the pattern of electrical activity in the cardiac ganglion. Thus, GABA does not mimic stimulation of the inhibitor nerves and appears to produce its effects at some site other than the cardioinhibitory pathway in the *Limulus* heart (Pax and Sanborn, 1967). If BOL is blocking the action of the inhibitor nerves by acting specifically at a junction in the cardioinhibitory pathway, and GABA is acting at some site other than this, then there should be no interaction between simultaneously applied BOL and GABA.

We have tested this in eight isolated hearts. One hundred ml. of saline containing GABA were initially perfused through each heart. After one-half hour of perfusion with drug-free saline, 100 ml. of 1.6×10^{-5} *M* BOL were perfused. This was followed immediately by 100 ml. of $1.6 \times 10^{-5}\ M$ BOL to which had been added the concentration of GABA previously given during the control perfusion. GABA at concentrations of 5, 10, and $50 \times 10^{-6}\ M$ was tested in this way against BOL at $1.6 \times 10^{-5}\ M$.

There is no apparent interaction between BOL and GABA so far as rate is concerned. The mean relative rate resulting from GABA perfusion prior to BOL treatment was 0.34 (mean decrease in rate, 19.7 beats per minute) while after BOL treatment it was 0.44 (mean decrease in rate, 15.4 beats per minute). In four out of eight hearts tested in this manner the relative rate resulting from GABA perfusion was higher after BOL treatment than before. In the other four it was lower. The mean difference between the relative rate prior to, and following BOL treatment was 0.10 (SD = 0.18). A "*t*" test for the difference between the relative rate before BOL treatment and after BOL treatment showed it to be non-significant ($P < 0.90$).

Table I

Summary of the responses of the Limulus heart to treatment with various inhibitors and antagonists

Inhibitor	5-HT	5-HT	GABA
Antagonist	BOL	Picrotoxin	BOL
Mean relative rate			
No. animals tested	4	4	8
Inhibitor alone	0.57	0.34	0.34
Inhibitor with antagonist	0.93	0.75	0.44
Difference ± 1 SD	0.36 ± 0.09	0.41 ± 0.18	0.10 ± 0.19
	$P < 0.01$	$P < 0.01$	$0.10 < P < 0.20$
Mean relative contraction strength			
No. animals tested	4	4	5
Inhibitor alone	0.41	0.51	0.67
Inhibitor with antagonist	1.04	0.26	0.65
Difference ± 1 SD	0.63 ± 0.21	−0.25 ± 0.11	−0.02 ± 0.07
	$P < 0.01$	$0.01 < P < 0.05$	$0.60 < P < 0.70$

Similarly, there is no apparent interaction between BOL and GABA with respect to strength of contraction. For five hearts the relative contraction strength resulting from GABA perfusion prior to BOL treatment was 0.67 while after BOL treatment it was 0.65. A "*t*" test for the difference between these means showed it not significant ($P < 0.90$).

The results of the various treatments are summarized in Table I.

Discussion

In the first paper of this series (Pax and Sanborn, 1964), we presented our reasons for believing that a chemical transmitter was involved in the inhibition of the neurogenic *Limulus* heart. At this point it is appropriate to examine the known and possible components of the *Limulus* cardioinhibitory system in order to visualize the sites at which chemical transmitters might operate.

Both the decapod crustacean cardiac ganglion and the *Limulus* cardiac ganglion possess two cell types (Heinbecker, 1936). The primary difference between the *Limulus* heart and the crustacean heart appears to be in the number of cells involved and it would seem reasonable to assume that the mechanism by which the rhythmic discharge is originated is common to both hearts (Maynard, 1955).

In the crustacean cardiac ganglion the burst is usually initiated by the smaller cells, the pacemakers. The larger cells are the major motor neurons (followers) and appear to be only relays which increase the number of impulses. Feedback, if any, from the followers to the pacemakers is small since only long subthreshold current pulses to the followers or a long series of follower cell impulses are necessary for modification of the rhythm of the pacemaker cells (Otani and Bullock, 1959). The inhibitor fibers make connections with both the pacemaker and the follower cells (Terzuolo and Bullock, 1958).

If the *Limulus* cardiac ganglion has an arrangement of functional units similar to the decapod heart, then we may diagram the inhibitory pathway as in Figure 10. Spontaneous rhythmic activity in the pacemaker cell (P) produces postsynaptic potentials in the follower cell (F). These postsynaptic potentials result in propagated action potentials which produce contraction of the myocardium (M). A block in transmission across the neuromuscular junction (C) would result in only a decreased strength of contraction. A block in transmission at junction "B" would give the same results.

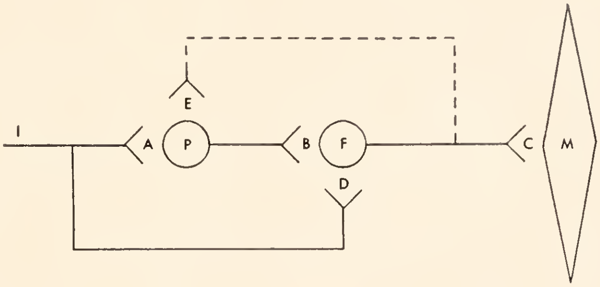

FIGURE 10. Diagram showing possible organization of the nerve cells and the cardioinhibitory connections in the cardiac ganglion of *Limulus*. Symbols explained in the text.

Activity in the inhibitor nerve through its action at junction "A" would produce a decrease in the rate of spontaneous bursting in cell "P" and thus cause a decrease in heart beat rate. Some lesser effect on contraction strength might occur if activity in the inhibitor also lessens the number of discharges in the pacemaker during any particular burst of activity. At junction "D," activity in the inhibitor nerve would produce a decrease in contraction strength by reducing the number of action potentials in the follower cell.

Turning now to the results of the experiments described here we find that 5-HT decreases both rate and strength of beating. This could be due to activation of junction "A" alone or of both junction "A" and "D." BOL blocks the action of the inhibitor nerves but does not otherwise disrupt heart function and thus probably acts at junction "A" alone or at both "A" and "D." Since this compound also blocks the action of applied 5-HT it appears probable that 5-HT acts at these same junctions.

Picrotoxin also blocks the action of the inhibitor nerves without otherwise markedly disrupting heart function. Thus it probably also acts at junction "A" or both "A" and "D." The rate-decreasing action of 5-HT is blocked by picrotoxin so again it would appear that 5-HT is acting at these same junctions. In contrast to the antagonism shown between BOL and 5-HT as far as strength-changing abilities are concerned, picrotoxin enhances the strength-decreasing ability of 5-HT. Such a pattern of responses could occur if junction "D" possesses pharmacological properties which are slightly different from those of junction "A."

GABA interacts with neither picrotoxin nor BOL. Thus it appears to act at neither junction "A" nor "D." It probably does not act at junction "B" or "C" since the rate-reducing effects of GABA are produced in the isolated ganglion and no change in burst parameters is noted with application of GABA. At this time we have no way of assessing the significance of feedback from the followers to the pacemakers (dashed line and junction "E" in Figure 10). Perhaps the major site of action of GABA is in this pathway.

Whether the endogenous inhibitory transmitter of the heart of *Limulus* is 5-HT or some related compound is open to question. Cogeners of 5-HT such as 5,6-dihydroxytryptamine, 6-hydroxytryptamine, or other substituted hydroxytryptamines have not been tested and may be as potent as 5-HT. In the crustacean heart 5-HT, 5,6-dihydroxytryptamine and 6-hydroxytryptamine are all potent cardiotropic agents and all have been detected in tissue extracts (Carlisle, 1956; Maynard and Welsh, 1959; Kerkut and Price, 1964; Belamarich and Terwilliger, 1966).

We wish to thank the National Science Foundation for supporting some aspects of these studies. Professor Tom S. Miya of the Purdue Department of Pharmacology has been generous with advice and contributed the BOL used in these studies.

SUMMARY

1. Heart rate in *Limulus* is slowed by 5-hydroxytryptamine (5-HT). The threshold for this inhibition is 4.9×10^{-8} *M*.

2. The strength of beat is also reduced in 5-HT solutions. The calculated threshold for this effect is 5.6×10^{-8} *M*.

3. Both of these effects are readily reversible.

4. Neither 5-hydroxytryptophan (10^{-4} *M*) or 5-hydroxyindole acetic acid (10^{-4} *M*) have any detectable effects on rate or strength of beating.

5. Applied to the isolated cardiac ganglion, 5-hydroxytryptamine (10^{-6} *M*) decreases the rate of rhythmic discharge, reduces the number of neurons discharging in each burst, and lessens the duration of each burst. All of these effects are also reversible.

6. Bromlysergic acid diethylamide (BOL), 1.6×10^{-5} *M*, decreases the ability of the cardioinhibitory nerves to influence heart rate.

7. BOL prevents the rate and strength changes engendered by exogenous 5-HT applied to the isolated heart.

8. Picrotoxin antagonizes the decrease in heart rate produced by application of 5-HT, but synergizes with 5-HT with respect to its strength-decreasing ability.

9. No interaction between BOL and γ-aminobutyric acid (GABA) could be demonstrated.

LITERATURE CITED

Belamarich, F. A., and R. Terwilliger, 1966. Isolation and identification of cardio-excitor hormone from the pericardial organs of *Cancer borealis*. *Amer. Zool.*, **6**: 101–106.

Burgen, A. S. V., and S. W. Kuffler, 1957. Inhibition of the cardiac ganglion of *Limulus polyphemus* by 5-hydroxytryptamine. *Biol. Bull.*, **113**: 336.

Carlisle, D. G., 1956. An indole-alkylamine regulating heart beat in Crustacea. *Biochem. J.*, **63**: 32–33 P.

Carlson, A. J., 1904. The nervous origin of the heart-beat in *Limulus* and the nervous nature of co-ordination in the heart. *Amer. J. Physiol.*, **12**: 67–74.

Carlson, A. J., 1905. The nature of cardiac inhibition with special reference to the heart of *Limulus*. *Amer. J. Physiol.*, **13**: 217–240.

Carlson, A. J., 1906. On the point of action of drugs on the heart with special reference to the heart of *Limulus*. *Amer. J. Physiol.*, **17**: 177–210.

Cooke, I. M., 1966. The site of action of pericardial organ extract and 5-hydroxytryptamine in the decapod crustacean heart. *Amer. Zool.*, **6**: 107–122.

Florey, E., and E. Florey, 1954. Über die mögliche Bedeutung von Enteramin (5-oxy-Tryptamin) als nervöser Aktions-substanz bei Cephalopoden und dekapoden Crustacean. *Z. Naturforsch.*, **9**: 58–68.

Gyermek, L., 1961. 5-Hydroxytryptamine antagonists. *Pharmacol. Reviews*, **13**: 399–439.

Heinbecker, P., 1936. Potential analysis of a pacemaker mechanism in *Limulus polyphemus*. *Amer. J. Physiol.*, **117**: 686–700.

Kerkut, G. A., and M. A. Price, 1964. Chromatographic separation of cardioaccelerators (6-HT and a mucopeptide) from *Carcinus* heart. *Comp. Biochem. Physiol.*, **11**: 45–52.

Mansour, T. E., E. W. Sutherland, T. W. Rall and W. Bueding, 1960. The effect of serotonin (5-hydroxytryptamine) on the formation of adenosine 3′, 5′-phosphate by tissue particles from the liver fluke, *Fasciola hepatica*. *J. Biol. Chem.*, **235**: 466–470.

Maynard, D. M., 1955. Activity in a crustacean ganglion. II. Pattern and interaction in burst formation. *Biol. Bull.*, **109**: 420–436.

Maynard, D. M., and J. Welsh, 1959. Neurohormones of the pericardial organs of Brachyuran Crustacea. *J. Physiol.*, **149**: 215–227.

Otani, T., and T. H. Bullock, 1959. Effects of presetting the membrane potential of the soma of spontaneous and integrating ganglion cells. *Physiol. Zoöl.*, **32**: 104–114.

Pax, R. A., and R. C. Sanborn, 1964. Cardioregulation in *Limulus*. I. Physiology of inhibitor nerves. *Biol. Bull.*, **126**: 133–141.

Pax, R. A., and R. C. Sanborn, 1967. Cardioregulation in *Limulus*. II. Gamma aminobutyric acid, antagonists and inhibitor nerves. *Biol. Bull.*, **132**: 381–391.

Terzuolo, C. A., and T. H. Bullock, 1958. Acceleration and inhibition in crustacean ganglion cells. *Arch. Ital. Biol.*, **96**: 117–134.

Welsh, J. H., and M. Moorhead, 1960. The quantitative distribution of 5-hydroxytryptamine in the invertebrates, especially their nervous systems. *J. Neurochem.*, **6**: 146–169.

SEMINAL LOSS IN REPEATEDLY MATED FEMALE AEDES AEGYPTI [1]

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Critical data are not available regarding the frequency of insemination of individual female *Aedes aegypti.* Males may inseminate as many as 6 or more females (Roth, 1948; Jones and Wheeler, 1965), and various authors have noted that females may mate several times. Subsequent matings, however, are of shorter duration than the first (Spielman, 1964). In a brief abstract, Vandehey and Craig (1958) indicated that multiple insemination may occasionally occur in caged populations.

Multiple matings in certain other mosquitoes have been studied more completely. Female *Culex pipiens* appeared to utilize sperm from only one of two genetically marked males with which they were confined (Kitzmiller and Laven, 1958; Spielman, 1956). On the other hand, *Anopheles gambiae* (Goma, 1963) and *A. quadrimaculatus* (French and Kitzmiller, 1963) occasionally accepted sperm from more than one male. The significance of these findings is limited in that caged populations may be abnormal in their mating behavior. Gillies' (1956) observation of multiple masses of semen in the genital atria of wild-caught *A. gambiae* females may indicate that multiple insemination occurs in nature.

The objective of the present study was to determine whether more than one semen mass is accepted and retained by female *A. aegypti.*

MATERIALS AND METHODS

A strain of *Aedes aegypti* obtained from Grand Bahama Island in 1965 was used. In addition, one experiment employed males of a genetically marked strain (Gold Mesonotum) obtained from Dr. George B. Craig, Jr. Larvae were reared in tap water at 22 ± 1° C. and fed Purina Rabbit Chow Pellets. Length of day was maintained at 16 hours and mosquitoes were manipulated at about the middle of the day.

Unless otherwise noted, males were 2–5 days of age when first mated. Mating of free-flying pairs occurred in glass lantern chimneys (18 × 10 cm.) at 22 ± 1° C. and 76 ± 2% R.H. The chimneys were rotated on their sides in order to induce continuous flight by the mosquitoes within. After mating began, rotation was stopped and duration of genital contact was noted. Mosquitoes were discarded if no copulation occurred within 2 minutes. To mate tethered specimens the female was etherized and glued to a slide. Males were anesthetized with gas (99% nitrogen and 1% hydrogen), decapitated, and held in vacuum forceps. Genitalia

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[1] This study was supported in part by U. S. Public Health Service Grant AI 00046 from the National Institute of Allergy and Infectious Diseases.

of these males were then brushed against those of the females until copulation occurred.

Mosquitoes were removed from chimneys with a breath-operated aspirator tube; those to be frozen were then blown into test tubes immersed in a mixture of alcohol and solid CO_2. Techniques of dissection, preparation of whole mounts and sectioning (employing Newcomer's fixative and hematoxylin-azure II-eosin stain) followed methods described by Spielman (1964). Techniques for transplanting tissue into mosquitoes have been described by Leahy and Craig (1965).

RESULTS

1. *Filling of the copulatory bursa*

The rate of inflation of the copulatory bursae of mating females was determined. Virgin pairs which were permitted to mate in lantern chimneys were removed and quick-frozen at − 70° C. at intervals after copulation began. Females were fixed while frozen, dissected, and mounted to permit a lateral view of the bursa. The outline of each bursa was sketched with a camera lucida (× 225) and the area of the sketch measured with a polar planimeter. (Measurement of the lateral profile of the bursa provided an adequate measure of degree of distention because inflation did not markedly affect the transverse dimension.) After 4 seconds of mating, bursae were approximately as distended as those in females that were allowed to mate without interruption (Fig. 1).

The duration of copulation necessary for the retention of semen after mating was studied in females whose mating was interrupted mechanically. This is in

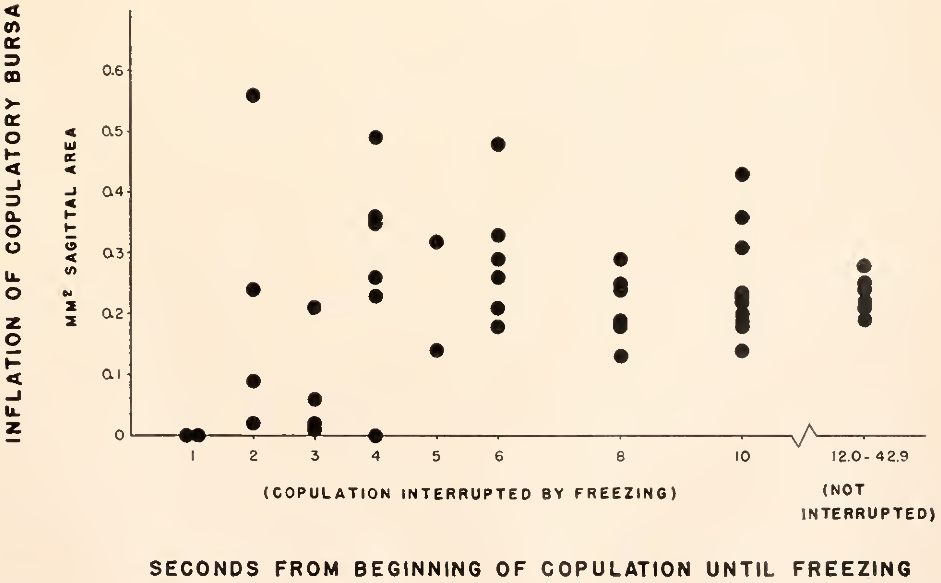


FIGURE 1. Rate of inflation of the copulatory bursa during mating. When copulation was not interrupted, females were frozen immediately after withdrawal of the male.

contrast to interruption by quick-freezing as practiced in the previous experiment. Five minutes after separation from the males, the females were chloroformed and dissected. Those that had mated for 6 seconds contained semen (Table I).

Subsequently, all experiments involving successive matings were done with females known to have mated for at least 6 seconds. Duration of copulation among virgin specimens generally exceeded 10 seconds; the mean for 128 pairs was 17.6 seconds (Table II).

2. *Behavior during successive matings*

The mating behavior of virgin and non-virgin females was compared. Single virgin females were placed with two males in lantern chimneys. Females were removed after copulation and held for varying periods of time before re-exposure to males. Females appeared to mate somewhat more readily when first exposed to males than during second exposure (Table II). Thus, nearly 90% of 3-day-old virgin females mated for 6 seconds or more while less than half mated a second time when re-exposed during the next week. Virgin females of comparable age mated readily. Abortive matings (*i.e.,* duration less than 6 seconds) occurred with greatest frequency in the previously-mated group. Of those females that mated, most did so during the first minute of exposure to males.

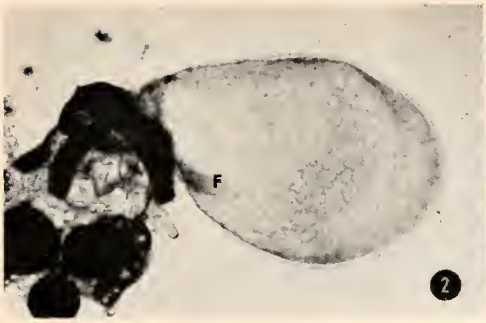
TABLE I

Presence of semen in female A. aegypti according to duration of interrupted mating

Duration of mating (seconds)	Proportion of females containing semen
1	0/2
2	3/10
3	2/6
4	4/9
5	4/5
6	6/6

3. *Appearance of the copulatory bursa after mating*

The copulatory bursa of freshly inseminated females was distended. Its contents included motile sperm and many clear globules intermixed with fine granules. Sperm were most numerous in a clear region at the periphery. The bursal walls, which were approximately 2 microns thick before mating, generally became vacuolated and as much as 20 microns thick after mating (Fig. 2). During the ensuing day, the bursa gradually lost most of its volume, globules disappeared, sperm became quiescent, and the bursal walls again became thin. Within the first 10 minutes after mating, the genital atrium contained a rapidly undulating mass of sperm. The sperm were directed toward the spermathecal eminence and the spermathecal vestibule, forming a flickering mass that later dispersed. A distended bursa, filled with motile sperm and clear globules, was accordingly taken as evidence of recent insemination.



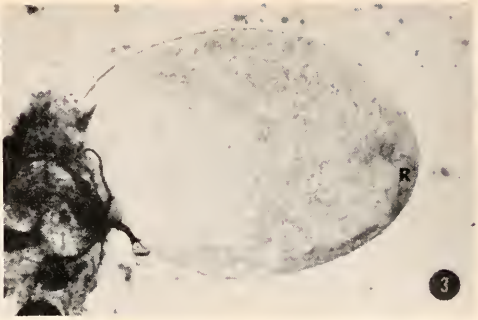


FIGURE 2. Copulatory bursa of female after being inseminated for the first time. Clear globules are present in the center of the semen mass; a swirling mass of sperm (F) is in the ventral portion of the bursal orifice and the bursal wall is thick and vacuolated.

FIGURE 3. Copulatory bursa of female that had mated twice and retained semen from both matings. Bursa was dissected after the second mating which followed one week after the first. The clear globules and swirling sperm of the second mating are present, together with the dark remnants of the initial semen mass (R). The wall of the bursa is thin and membrane-like.

4. *Presence of semen after mating*

The frequency with which virgin females became inseminated was studied in females of various ages. Those that mated for at least 6 seconds were chloroformed and the copulatory bursae examined within 5 minutes of mating. Semen was almost invariably present after young virgin females mated (Table III).

In contrast, previously mated females generally did not contain fresh semen after second mating (Table III). This effect was especially marked when one or

TABLE II

Readiness to mate and duration of mating of individual females during consecutive or single exposures to males

	Consecutively mated group		Control; single-mated group
	First mating of 3-day-old females	Second mating at 5–10 days of age	First mating of 5–10-day-old females
Number of females individually exposed to males for 2 minutes	128	111	29
% of females mating for 6 seconds or more	87.5	46.9	100
Mean number of contacts of less than 6 seconds per mated female	1.5 ± 0.3*	2.5 ± 0.4	1.7 ± 0.6
Mean seconds until mating (6 seconds or more) commenced	35.1 ± 4.2	45.0 ± 4.7	47.5 ± 6.5
Mean seconds duration of mating	17.6 ± 0.5	12.6 ± 1.3	19.4 ± 1.3

* Standard error.

two days had elapsed between the first and second matings. The bursae of females that retained semen from the second mating invariably contained identifiable remnants of the first seminal mass (Fig. 3). The first mass was darker than the second, clearly demarcated, and located apically. It was thus possible in each instance to confirm that prior insemination had occurred. Furthermore, in more than half (8 out of 14) of the twice-inseminated females, the walls of the bursa remained thin. The bursal walls of females that were mated for the first time almost invariably (95 out of 96) became thick.

TABLE III

Comparison of retention of semen by females after second mating with that observed in once-mated control group of comparable age

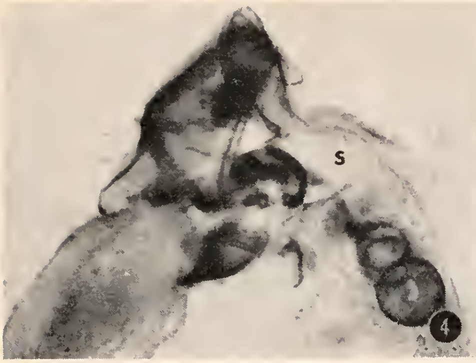
Previously mated			Control groups of same age with no previous mating	
Time to second mating	No. females	% retaining semen after second mating	No. females	% retaining semen after initial mating
½–4 hrs.	22	27	22	100
5 hrs.	16	19	16	100
1 day	15	0	14	100
2 days	27	7	25	96
1 week	27	11	15	93
2 weeks	25	24	15	93
3 weeks	12	8	9	78

5. *Loss of semen following second mating*

The absence of fresh semen in most females following second mating suggested that insemination might not have occurred despite observed genital contact of sufficient duration to ensure effective mating of virgin females. Accordingly, mosquitoes were frozen during copulation to compare semen transfer in first and second matings. Eighteen females were frozen during their first mating; of these, 14 were prepared as whole mounts and 4 were sectioned. Eleven additional females were re-mated two days after initial mating and similarly frozen; 7 of this group were mounted whole and 4 sectioned. Semen was present in the copulatory bursae of all 29 females in both groups (Fig. 4). Thus, insemination of virgin females and of previously mated females occurred with equal frequency.

Copulation of virgin and of once-mated females was compared. Genital union in all cases was firm and corresponded closely to descriptions by Spielman (1964).

The presence of semen in the copulatory bursa during second mating and its absence after separation indicated that semen must have been expelled following the mating. This process is illustrated by the female in Figure 5, one which was fortuitously frozen as it was separating from the male. The paraprocts and claspers of the male were in normal copulating position and in contact with the female. The aedeagus, however, was retracted. The female's genital parts, too, were in copulating position, but the copulatory bursa was contracted and a mass of semen was present between the genital lips. Taken together, these observations suggest that the seminal mass was expelled from the copulatory bursa following withdrawal of the aedeagus.



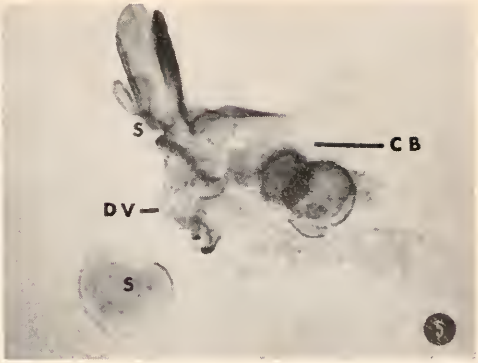


FIGURE 4. Whole mount of pair that was frozen during copulation. Although the aedeagus of the male (left) is only partially extended, the copulatory bursa is distended with semen (S). The spermathecae contain sperm from a previous mating.

FIGURE 5. Whole mount of copulating female that was frozen as the aedeagus was withdrawn. The copulatory bursa (CB) is empty and a mass of semen (S) was present between the genital lips and adherent to the post-genital plate. Sperm from a previous mating is present in the spermathecae but not visible in the figure.

Seminal expulsion following insemination of previously mated females was also observed directly when mosquitoes were mated manually. Ten 5-day-old females, mated two days previously, were tethered and brought into contact with virgin males. A mass of semen was visible externally on 9 of the females as the pairs were separated 10–20 seconds later. The tenth female contained semen as did all 10 of a group of virgin females that were similarly mated as controls.

6. *Loss of semen following mating of virgin females implanted with male tissue*

The preceding observations suggested that loss of semen in twice-mated females might be due to some attribute of semen transferred to the female during first copulation. To explore this possibility, organs removed from 3- or 4-day-old virgins were implanted in the thoraxes of 3- or 4-day-old virgin females as follows: male accessory gland (Group A), testis (Group B), and ovary (Group C). Controls included normal female virgins of the same age (Group D) and normal females

TABLE IV

Seminal retention after mating in virgin females that had received tissue implants 2 days previously

Group	Tissue implanted	Females	
		Number	% retaining semen
A	Male accessory gland	28	21
B	Testis	16	81
C	Ovary	10	100
D	No implant (virgin)	10	100
E	No implant (mated 2 days previously)	22	14

which had mated at the time that groups A–C received implants (Group E). All were individually exposed to males two days after groups A–C received implants. The post-mating results (Table IV) showed that females in groups A and E tended to lose semen, that loss of semen was less frequent in Group B, and that semen was retained by all mosquitoes in Groups C and D. The findings suggest that male accessory gland and, to a lesser extent, the testis contain materials that may be responsible for seminal loss.

The effect upon the wall of the copulatory bursa produced by implantation of male accessory glands was studied in 10 virgin females which were examined approximately one hour after receiving the implants. In 9 the bursal walls were vacuolated and more than 2 microns thick; in two of these the bursal walls were highly vacuolated and about 20 microns thick. By comparison, the bursal walls were non-vacuolated in 10 females implanted with ovarian tissue, and in only one of these females was the bursal wall thicker than 2 microns.

TABLE V

Effect of blood feeding upon seminal retention in non-virgin females. At beginning of experiment, females were mated, then fed blood and re-mated after 1 week. In control groups, initial mating and/or blood feeding was omitted

Treatment at beginning of experiment	Females	
	Number	% retaining semen after subsequent mating
Mated. Blood-fed	22	28
Mated. Not exposed to host	23	13
Not exposed to males. Blood-fed	14	100
Not exposed to males. Not exposed to host	17	100

7. *Utilization of sperm received in second mating*

Although semen was occasionally retained by twice-mated females, it was not known whether the sperm from the second mating were utilized. This was tested by mating genetically marked (Gold Mesonotum) males with wild-type females 5 hours after the females had first been inseminated by males of their own type. Although Gold Mesonotum has frequent penetrance in heterozygous females (Craig and Vandehey, 1962), it is not completely dominant, and the resulting families would contain fewer marked females than actual heterozygotes. Of 22 families produced, 4 contained females of both genetic types. Thus, semen retained from a second mating is capable of fertilizing eggs.

8. *Effect of blood feeding upon seminal retention in non-virgin females*

The influence of blood meals and resulting ovarian development on retention of semen in previously mated females was studied. Three-day-old virgin females were mated individually in lantern chimneys; one hour later they were permitted to feed on a guinea pig. Blooded mosquitoes were re-mated one week later. In control groups of similar virgin females, initial mating, the blood meal, or both, were omitted. Raisins were provided as supplemental food. Retention of semen was universal among females mated for the first time, regardless of food regimen (Table V). Retention after second mating, however, appeared to be somewhat enhanced by blood feeding.

Discussion

In most insects, males transfer sperm to the female by means of spermatophores from which the sperm escape after the copulating pair separates (Khalifa, 1949; Davey, 1960). Other animals may instead possess seminal gels that harden within the female, and "it is sometimes supposed that in vertebrates such plugs assist insemination by preventing loss of semen from the female genital tract" (Hinton, 1964, p. 96). Diptera do not have spermatophores, and it seems likely that some special device may aid in the retention of semen after mating. Copulation in *A. aegypti* is accomplished through the superficial apposition of genital parts, semen being extruded into a chamber formed by the mating pair (Spielman, 1964). This arrangement might result in loss of semen as the male withdraws unless the seminal mass becomes altered in some way during or immediately after insemination. Indeed, the appearance of clear globules in the copulatory bursa during the first minute of a first mating may be associated with such a change. In addition, semen appears to be expelled unless held within the female by the aedeagus of the male for a few seconds. The formation of a "mating plug" following insemination of various mosquito species has been described (Gillies, 1956; Lum, 1961).

Loss of semen after a second mating occurred more frequently than after the first, especially if the period between matings exceeded a few hours. This suggests that prior mating may interfere with the normal reaction of the female's genital tract to semen. Under these conditions, the mass of semen flows freely and is lost when the female's genital orifice is vacated by the aedeagus.

Vacuolization of the bursal wall is one reaction of the female's genital tract to the semen (Spielman, 1964; Jones and Wheeler, 1965). That vacuolization frequently did not occur following a second mating of female *A. aegypti* suggests that the wall of the bursa may have some role in seminal retention. However, vacuolization is not an absolute prerequisite to seminal retention, because in some females that retained semen, vacuolization was not observed.

Loss of semen deposited in a second mating appears to be due to some component of the semen of the first mating, and the male's accessory glands, which elaborate a major portion of the semen, are the probable source of this factor. There need not be direct contact with the bursa, for seminal loss and vacuolization of the bursal wall occurred after the male tissue was implanted in the thoraxes of virgin females. Interestingly, male accessory glands have also been shown to contain a material that enhances oviposition in *A. aegypti* females (Leahy and Craig, 1965).

The present observations indicate that effective multiple insemination of female *A. aegypti* may be infrequent in nature. Indeed, if the second mating does not occur during the same day as the first, it appears unlikely that semen from both would be retained.

Summary

1. The effectiveness of mating of female *Aedes aegypti* that had previously been mated was compared to that of virgin females. Non-virgin females mated

less readily than virgin females and copulation was of somewhat shorter duration. Genital union was firm, and insemination occurred in both virgin and non-virgin females, yet semen was generally not retained in the copulatory bursa of females that had previously been mated. This effect was most evident when one or two days had elapsed between matings. Multiple insemination, with utilization of sperm from both matings, however, was occasionally effective when less than 5 hours separated the first and second matings.

2. Factors derived from the accessory glands of the male and, to a lesser extent, the testes appeared to induce this loss of semen.

3. It was suggested that semen normally gels during mating and that loss of semen following second mating may result from a defect in this process.

4. These data indicate that female *A. aegypti* in nature may normally utilize sperm from but one male.

ADDENDUM

Recently, George[1] observed female *A. aegypti* that were sequentially mated to irradiated and to non-irradiated males. He suggested that, "copulation may occur repeatedly, (but) the only effective one is the first" (p. 85).

LITERATURE CITED

CRAIG, G. B., AND R. C. VANDEHEY, 1962. Genetic variability in *Aedes aegypti* (Diptera: Culicidae). I. Mutations affecting color pattern. *Ann. Ent. Soc. America*, **55**: 47–58.

DAVEY, K. G., 1960. The evolution of spermatophores in insects. *Proc. Roy. Ent. Soc. London (A)*. **35**: 107–113.

FRENCH, W. L., AND J. B. KITZMILLER, 1963. Tests for multiple fertilization in *Anopheles quadrimaculatus*. *Proc. New Jersey Mosq. Exterm. Ass.*, **50**: 374–380.

GILLIES, M. T., 1956. A new character for the recognition of nulliparous females of *Anopheles gambiae*. *Bull. World Health Org.*, **15**: 451–459.

GOMA, L. K. H., 1963. Tests for multiple insemination in *Anopheles gambiae* Giles. *Nature*, **197**: 99–100.

HINTON, H. E., 1964. Sperm transfer in insects and the evolution of haemocoelic insemination. *In:* Insect Reproduction. *Symp. Roy. Ent. Soc. London*, **2**: 95–107.

JONES, J. C., AND R. E. WHEELER, 1965. Studies on spermathecal filling in *Aedes aegypti* (Linnaeus). I. Description. *Biol. Bull.*, **129**: 134–150.

KHALIFA, A., 1949. Spermatophore production in Trichoptera and some other insects. *Trans. Roy. Ent. Soc. London*, **100**: 449–479.

KITZMILLER, J. B., AND H. LAVEN, 1958. Tests for multiple fertilization in *Culex* mosquitoes by the use of genetic markers. *Amer. J. Hyg.*, **67**: 207–213.

LEAHY, SR. M. G., AND G. B. CRAIG, JR., 1965. Accessory gland substance as a stimulant for oviposition in *Aedes aegypti* and *A. albopictus*. *Mosq. News*, **25**: 448–452.

LUM, P. T. M., 1961. The reproductive system of some Florida mosquitoes. II. The male accessory glands and their role. *Ann. Ent. Soc. America*, **54**: 430–433.

ROTH, L. M., 1948. A study of mosquito behavior. An experimental laboratory study of the sexual behavior of *Aedes aegypti* (Linnaeus). *Amer. Midl. Natural.*, **40**: 265–352.

SPIELMAN, A., 1956. The inheritance of autogeny in the *Culex pipiens* complex of mosquitoes. Thesis, Johns Hopkins School of Hygiene and Public Health.

SPIELMAN, A., 1964. The mechanics of copulation in *Aedes aegypti*. *Biol. Bull.*, **127**: 324–344.

VANDEHEY, R. C., AND G. B. CRAIG, 1958. Multiple fertilization demonstrated in *Aedes aegypti*. *Bull. Ent. Soc. America*, **4**: 102.

---

[1] George, J. A., 1967. Effect of mating sequence on egg-hatch from female *Aedes aegypti* (L.) mated with irradiated and normal males. *Mosq. News*, **27**: 82–86.

CAROTENOID PIGMENTS IN THE CELLULAR SLIME MOLD, DICTYOSTELIUM DISCOIDEUM[1, 2]

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Five distinct stages of development may be recognized in the life-cycle of the cellular slime mold, *Dictyostelium discoideum* (Bonner, 1944; Raper, 1937, 1939, 1940). The first four stages are relatively colorless, but the fifth, or fruiting body, stage is marked by a change in the color of the sori from pale buff to bright lemon yellow. Since a change in color in an organism may reflect changes in metabolic events, the nature of the pigments was investigated to determine: (1) whether this color transition represents *de novo* synthesis by the spore cells or the mere accumulation of substrate pigments from the medium, as suggested by Whittingham and Raper (1956); (2) the nature of the pigment; (3) the effect of diphenylamine, which specifically inhibits carotenogenesis (Goodwin, 1952, 1954; Haxo, 1955; Kharasch, 1936; Turian, 1950; Zalokar, 1957).

METHODS AND MATERIALS

Culture procedures and harvesting of tissue

All cultures of *D. discoideum* and the bacterial associate, *Escherichia coli*, were maintained on an agar medium (Bonner, 1947) in Petri plates. Cultures were incubated in darkness at 22° C. in an environment ranging from 55 to 90% relative humidity. Following various intervals of incubation, cultures of *D. discoideum* were harvested for dry weight determination and pigment assay. Cultures were scraped into tared flasks and weighed wet. An aliquot of known wet weight was removed for dry weight determination and the remaining wet sample was extracted for pigment. The aliquot for dry weight determination was freed of *E. coli* in a 0.55 M/0.95 M sucrose gradient, washed, dried, and weighed. This procedure permitted calculation of the dry weight of the sample used for pigment extraction.

In order to extract the pigment, cultures of the desired stages were scraped into 95% ethanol, allowed to stand several hours, and filtered. The residue, from greyish-white to pale yellow in color, was discarded, and the yellow filtrate (ethanol extract) was stored in darkness at 10° C. Extracts obtained by this procedure were either assayed for pigment as described below or purified further.

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[1] Presented to the Graduate Council of the University of Florida in partial fulfillment of the requirements for the degree of Master of Science (S. O. S.).

[2] This investigation was supported in part by a Public Health Service Research Career Programs Award 5-K3-HD-15,780 from the National Institute of Child Health and Human Development and Research Grants E-1452 and GM-10138 from the National Institutes of Health (J. H. G.).

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Assay of pigment

The optical density (O. D.) at 390 mμ of ethanol extracts was proportional to the O. D. at 390 mμ of the more purified preparations described later, and was suitable for gross quantitative assay of pigment.

Purification of pigment for analysis

When large quantities of pigment were needed for chemical analyses, only cultures of mature sorocarps were harvested, and were extracted as described previously. Cultures containing only *E. coli* and nutrient agar contained no pigment and were not further studied. The ethanol extract was saponified with 10% KOH (w/v) at 65° C. for 2 hours. Following hydrolysis, the pigment solution was transferred to a separatory funnel with an equal volume of diethyl ether and sufficient water to effect separation of the two phases. Acetic acid was added to transfer all pigment into the ether phase. The epiphase was then washed with water, dried over anhydrous Na_2SO_4, transferred to an Erlenmeyer flask, and taken to dryness under reduced pressure (Residue I). Small portions of hexane were added to Residue I and then decanted from the flask until further additions of solvent remained colorless. The hexane extracts were combined, reduced in volume under vacuum, and poured onto a powdered sucrose column. The column was first developed with hexane, and then washed successively with diethyl ether and methanol. The major yellow fraction was eluted with ether, and is designated Fraction I. The hexane eluate, which was also yellow, was rechromatographed on MgO:Celite (1:1). Several pigments were subsequently eluted from this column with hexane and ether, but the very small quantities present precluded further analysis. Residue I was subsequently extracted with ether, and the ether-soluble pigments were also chromatographed on powdered sucrose. Developing the column with ether eluted a pigment designated Fraction II-a. The column was then washed with methanol, eluting Fraction II-b. Following removal of the hexane and ether-soluble pigments from Residue I, the remaining pale yellow residue was taken up in methanol (Fraction III).

Chemical analyses of fractions

The presence of an acidic function was tested by noting differences in the distribution behavior of the salt and the free acid between two solvents (Fox, 1953; Zalokar, 1957). The absorption spectra of the acidic and basic forms of the pigment were also compared (Zalokar, 1957).

Several qualitative color tests were used to detect polyene structure. Fractions I through III were taken to dryness, and a drop of concentrated H_2SO_4 was added to the residues. In addition, concentrated H_2SO_4 was layered under ether solutions of Fractions I and II-a (Karrer and Jucker, 1950). A few crystals of dithionite, a reducing agent, were added to Fractions I and II-a in ether and to Fractions II-b and III in methanol. Color changes were noted. Antimony trichloride was added to chloroform solutions of Fractions I and II-a (Carr and Price, 1926; Karrer and Jucker, 1950). This reaction could not be carried out on Fractions II-b and III because they were insoluble in chloroform.

Spectrophotometric analyses

All spectral data were obtained with either a Beckman DK-2 or a Bausch and Lomb Spectronic 505 recording spectrophotometer.

The effects of diphenylamine

Culture media were prepared by adding to the agar a stock solution of 10^{-2} *M* diphenylamine (DPA) in 95% ethanol to give concentrations of DPA from 5×10^{-6} *M* to 5×10^{-5} *M*. Controls were prepared by adding the appropriate volume of ethanol to the culture media. Plates were then inoculated, incubated for various intervals, and examined for relative number of sorocarps and intensity of coloration.

RESULTS

Studies relating pigment concentration to developmental stage

The course of pigmentation is shown in Figure 1. The concentration of pigment was low initially and did not change appreciably for 60 hours. It then increased slowly until fruiting began at about 72 hours. At 84 to 96 hours, fruiting was morphologically complete and the pigment concentration continued to increase. Major pigment accumulation did not occur until after fruiting was complete.

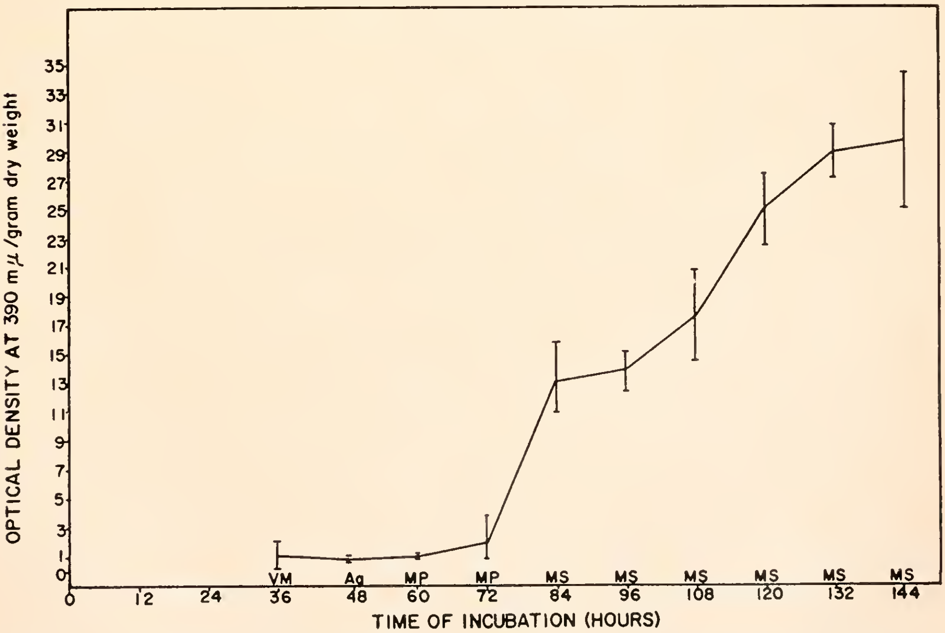

FIGURE 1. Increase in pigment concentration during development. VM = Vegetative myxamoebae. Ag = Aggregates. MP = Migrating pseudoplasmodia. MS = Mature sorocarps.

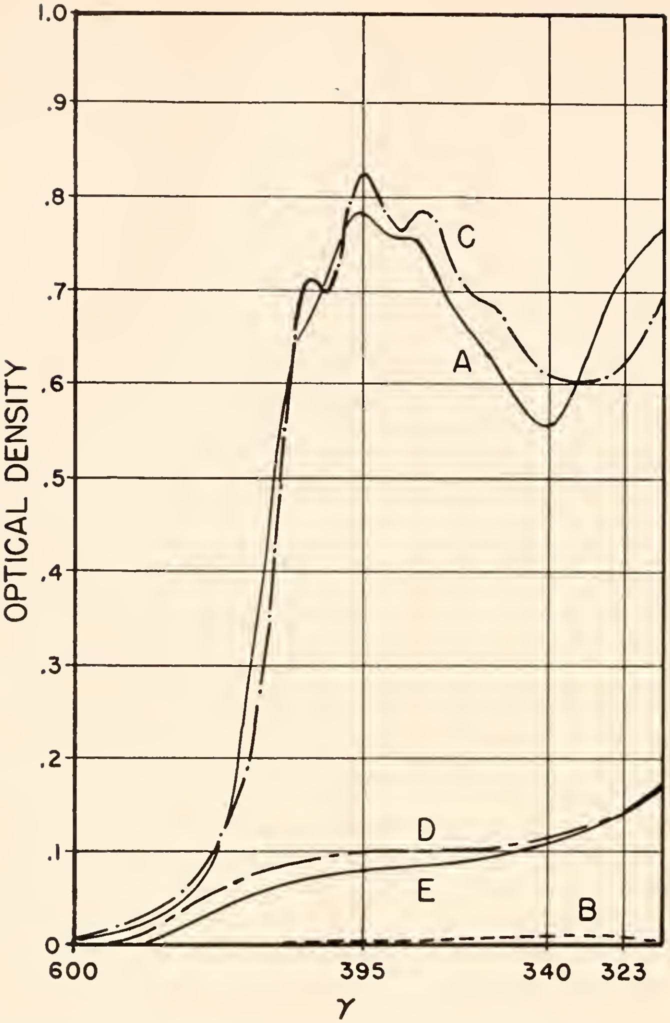

Figure 2. Absorption spectra of *D. discoideum* and *E. coli* extracts. A = *D. discoideum* ethanol extract. B = *E. coli* ethanol extract. C = *D. discoideum* ether phase. D = *E. coli* ether phase. E = Control hydrolysate.

Comparison of spectra of D. discoideum, E. coli, *and nutrient agar ethanol extracts*

The ethanol extracts of *D. discoideum, E. coli,* and nutrient agar were examined spectrophotometrically. The *D. discoideum* extract showed a peak at 398 mμ with shoulders at 414 mμ and 381 mμ. The spectra of the *E. coli* and agar extracts showed no absorption of the visible region (*cf.* Curves A and B, Figure 2). Further, the ether phases obtained after hydrolysis of ethanol extracts of *D. discoideum, E. coli,* and agar were compared. As shown in Figure 2, the strong absorption band seen in the ethanol extracts of *D. discoideum* was resolved to yield absorption maxima at 417 mμ, 397 mμ, and 377 mμ (Curve C). On the other hand, the spectra of the colorless *E. coli* and agar extracts were essentially the same as that of the control.

Spectral characteristics of pigment fractions

The absorption maxima of the pigment fractions are summarized in Table I.

Chemical tests

Test for an acidic function

Partitioning an ether solution of Fraction I against 2 N NaOH moved the pigment to the interface where it was visible as a yellow layer. Addition of methanol distributed the pigment between the two phases. Acidification restored the pigment to the ether phase.

TABLE I

Comparison of literature and experimental maxima at λ_1

Compound	Solvent	Maxima at λ_1				
zeta-carotene[a]	Hexane	425	400	378	360	295
zeta-carotene[b]	Petroleum ether	418	396	376		
OH-zeta-carotene[b]	Petroleum ether	417	396	376		
di-OH-zeta-carotene[b]	Petroleum ether	420	397	378		
θ-carotene[c]	Not specified	421	397	375.5		
Fraction I	Diethyl ether	418	397*	378*	360	338
Fraction II-a	Diethyl ether	418	396*	375*		
Fraction II-b	Methanol	414	397*	378		
Fraction III	Methanol		397			
Calculated with Kuhn's formula	None	408				

* Indicates λ_{max}.
[a] Nash, 1945.
[b] Jensen, 1958.
[c] Haxo, 1955.

When the ether solution of Fraction II-a was partitioned against alkali, the pigment was distributed between the two phases. Acidification moved all pigment into the epiphase. Hence Fraction II-a may contain at least two components, only one of which has an acidic group.

When partitioned against ether and alkali, all pigments in Fractions II-b and III were hypophasic. Addition of methanol moved very little pigment into the epiphase. Acidification moved the pigments into the epiphase. Therefore, the pigments of Fractions II-b and III possess acidic functions.

Test for an acidic function in conjugation with the chromophore

The positions of the maxima of Fractions I, II-a, and II-b in the visible region were not altered by pH. The intensity of absorption, however, increased in basic solutions and decreased in acidic solutions.

The maximum of Fraction III occurred at 392 mμ in alkaline methanol but was at 397 mμ in acidic methanol. The absorption increased in alkaline methanol and decreased in acidic methanol. The spectral shift of 5 mμ is consistent with the data for neurosporaxanthin (Zalokar, 1957) and indicates that the acidic function is conjugated with the chromophore.

Qualitative tests for polyene structure

The tests in Fractions I and II-a were all weakly positive; treatment with H_2SO_4 produced traces of blue which rapidly gave way to relatively stable brown colors. The Carr-Price reaction yielded deep bluish-orange colors. The color of these fractions was not entirely abolished by dithionite, although absorption in the visible disappeared. On the other hand, the brilliant blue colors obtained by treating Fractions II-b and III with H_2SO_4 (Haxo, 1949; Karrer and Jucker, 1950), and the complete decolorization of these two fractions by dithionite showed polyene structures.

TABLE II

The effect of various concentrations of DPA upon mature sorocarp formation and pigment synthesis in D. discoideum

Concentration of DPA in media	Relative number of sorocarps	Relative pigment concentration
5×10^{-5} *M*	None	—
4×10^{-5} *M*	Very few	Colorless
3×10^{-5} *M*	Not abundant	Colorless to pale yellow
2×10^{-5} *M*	Abundant	Colorless to pale yellow
1×10^{-5} *M*	Abundant	Pale yellow
5×10^{-6} *M*	Abundant	Only slightly less yellow than controls
Ethanol control	Abundant	Bright lemon yellow
Control	Abundant	Bright lemon yellow

The results suggest that Fractions I and II-a contained carotenoid pigments, with, however, colorless impurities and non-carotenoid pigments of unknown nature. The data point to many similarities between these non-carotenoid pigments and the lipofuschins described by Fox (1953). Unquestionably the pigments in Fractions II-b and II are carotenoids.

The effect of diphenylamine on pigment synthesis

The effects of several concentrations of DPA upon pigment synthesis are given in Table II. At 2×10^{-5} *M* to 3×10^{-5} *M* it significantly inhibited pigment synthesis without interfering with growth. This concentration range was very critical. Growth was severely limited at 5×10^{-5} *M* DPA, but in the presence of 5×10^{-6} *M* DPA the mature sorocarps were practically indistinguishable from the controls.

DISCUSSION

Whittingham and Raper (1956) have suggested that pigmentation in *D. discoideum* sori depends upon environmental factors, such as the substratum or the bacterial associate upon which the slime mold feeds. Other studies have established that sorocarp color may be influenced by the incorporation of vital dyes (Bonner, 1952) or pigmented foodstuffs (Raper, 1937). In these instances pigmentation occurs as a result of the accumulation and retention of soluble or particulate exogenous pigment.

This investigation indicates that sorocarp pigmentation arises by *de novo* synthesis. Absorption spectra of ethanol extracts of *D. discoideum* showed in the visible region a characteristic peak which was absent from ethanol extracts of both *E. coli* and nutrient agar (Fig. 2). Further, a sharp separation exists between the feeding period and the morphogenetic phase of the life cycle (Bonner, 1947, 1959). Although food intake ceases at the beginning of aggregation (Bonner, 1959), the colorless pseudoplasmodia could be transferred to a non-nutrient agar surface where pigmented fruiting bodies subsequently occurred. Further evidence that the yellow pigment is not merely accumulated was obtained from the studies relating pigment concentration to development (Fig. 1). At the time pigment appears in significant quantities, the spore cells are supported in the air by a stalk, and thus are removed from immediate contact with any exogenous supply of pigment.

These pigments are mainly carotenoids. At a suitable concentration, DPA, a well known inhibitor of carotenoid synthesis, either decreased or completely inhibited pigmentation without affecting growth. In addition, the massive bands in the visible region of the absorption spectra are characteristic of carotenoid pigments. This band, believed to arise from the oscillation of pi electrons from one end of the conjugated polyene structure composing the chromophore to the other (Dale, 1954), often exhibits fine structure usually manifested by three maxima or two maxima and a shoulder. The peak with the highest intensity is referred to as the λ_{max}, while the whole band, including its fine structure, is referred to as the fundamental band or λ_1 (Dale, 1954; Zechmeister, 1960). For a given solvent, the position of this band and its degree of fine structure depend upon the length of the chromophore (Zechmeister, 1960). One of the most important determinants of the length of the chromophore and hence the position of λ_1 is the number of conjugated double bonds. This relationship has been worked out both theoretically and empirically so that by the position of λ_1, one can estimate rather closely the number of conjugated double bonds in the chromophore. From curves relating the number of conjugated double bonds and maxima at λ_1 (Dale, 1954; Nash, 1948), it was estimated that pigments in Fractions I, II-a, II-b, and III possessed seven conjugated double bonds. Then, Kuhn's empirical formula was used to calculate the wave-lengths near which a system with seven double bonds should display maxima (Table I). Kuhn's formula (Dale, 1954) is:

$$\lambda_s = \frac{157\ \text{m}\mu}{\sqrt{1 - 0.922 \cos\left(\frac{\text{pi}(s)}{n+1}\right)}}$$

where n = the number of conjugated double bonds and s = the band order. It can be seen that close agreement exists between observed and calculated values at λ_1, despite the dependence of the position of this band upon both the solvent and the presence or absence of an oxygen atom conjugated with the chromophore. The observed maxima at λ_1 are compared with literature values of known carotenoids in Table I.

The evidence that these pigments are acidic is of interest, for relatively few acidic carotenoids have been described. In the native state these pigments exhibited limited solubility in water. It is unlikely that combination with protein contributed in any substantial way to this water-solubility, for a dramatic spectral shift (Fox, 1948) was never observed when the pigments were subjected to procedures which would hydrolyze a protein moiety and liberate pigment. On the other hand, esterification with a sugar residue may be an important factor in conferring this limited water-solubility. Until the pigments were hydrolyzed, no amount of acid forced the pigments from the ethanol extract into the ether phase.

The partition behavior of these pigments was not studied extensively. However, the insolubility of Fractions II-b and III in such non-polar solvents as hexane, benzene, and carbon disulfide suggested the presence of polar groups. The possibility that these pigments were xanthophylls is of interest with respect to the observed DPA inhibition. Haxo (1955) reported that DPA inhibited formation of xanthophylls in *Mycobacterium phlei,* and Turian and Haxo (1952) found this inhibition to be most marked at the terminal synthetic steps, *i.e.,* at the conversion of neutral hydrocarbons into acidic compounds.

The evidence that these pigments are carotenoids and that they contain seven conjugated double bonds leads to the conclusion that they probably belong to the zeta-carotene series.

Functional significance of these pigments should be explored. Most attempts to establish a function of carotenoids in fungi have pointed to their mediating photokinetic responses (Goodwin, 1954). Migrating pseudoplasmodia in *D. discoideum* exhibit a strong positive photoactic response (Bonner, 1952); and Francis (1964) has recently shown this action spectrum to have a major peak near 425 mμ and a minor peak near 550 mμ. He found that the absorption spectrum of "slime" from spore heads also peaked about 425 mμ. On the basis of these two findings, he has suggested that the slime sheath may contain the receptor system for phototaxis. The absorption spectrum of sori "slime" which Francis has reported is similar to the spectra of pigments studied in this investigation, and it seems plausible that these components are identical. Although Francis has demonstrated a phototactic response in the migrating pseudoplasmodia, he did not study the absorption spectra of this stage. In the present study no absorption peak near 395 mμ, and consequently no yellow pigment, was found in migrating pseudoplasmodia. In order to demonstrate an association between phototaxis and a pigment, one should be able to correlate the absorption spectrum maximum with the same developmental stage as that in which the phototactic response occurs. Evidence obtained by other investigators (Goodwin, 1952, 1954) suggests that in some instances as little as 1–2% of the usual carotene content may be sufficient for phototactic action; or alternatively, that the more saturated polyenes mediate a photokinetic response. Another possibility is that the pigment and phototactic response have nothing to do with each other.

There is a great deal of circumstantial evidence in the literature pointing to, but never specifically defining, a reproductive function for carotenoids. In *D. discoideum,* carotenoids accumulate in the spores, *i.e.,* in the structures directly concerned with reproduction. The pigmentation reaches a peak after fruiting and sharply decreases in the vegetative phase. These findings are similar to those of other investigators (Emerson and Fox, 1940; Fox, 1948; Goodwin, 1950; Murneek, 1934) which indicate that the highest concentrations of carotenoids in plants and animals are found in tissues and secretions associated with reproduction, and suggest that carotenoids for some reason may be associated with reproduction in *D. discoideum* although no conclusions about their function can be drawn from the present evidence.

We are extremely indebted to Dr. James A. Olson of the Department of Biochemistry and Dr. Robert M. DeWitt of the Department of Zoology, University of Florida for their invaluable suggestions, advice and aid during the course of this investigation.

Summary

1. The lemon-yellow pigmentation in the mature sori of the cellular slime mold, *Dictyostelium discoideum,* was shown to arise by *de novo* synthesis and not by accumulation from an exogenous source. Pigment synthesis reached a peak after fruiting and then sharply declined in the vegetative phase.

2. The major pigments appeared to be related to the zeta-carotenes. Inhibition of pigment synthesis by diphenylamine, which specifically inhibits carotenogenesis, indicated the pigments were carotenoids. Chemical and spectral analyses of the pigments indicated polyene structures with seven conjugated double bonds.

3. Most of the pigments contained acidic functions. The acidic function of one pigment appeared to be conjugated with the chromophore.

LITERATURE CITED

Bonner, J. T., 1944. A descriptive study of the development of the slime mold *Dictyostelium discoideum. Amer. J. Bot.,* **31**: 175–182.

Bonner, J. T., 1947. Evidence for the formation of cell aggregates by chemotaxis in the development of the slime mold *Dictyostelium discoideum. J. Exp. Zool.,* **106**: 1–26.

Bonner, J. T., 1952. The pattern of differentiation in ameboid slime molds. *Amer. Nat.,* **86**: 79–89.

Bonner, J. T., 1959. The Cellular Slime Molds. Princeton University Press, Princeton, New Jersey.

Carr, F. H., and E. A. Price, 1926. Color reactions attributed to vitamin A. *Biochem. J.,* **20**: 497–501.

Dale, J., 1954. Empirical relationships of the minor bands in the absorption spectra of polyenes. *Acta Chem. Scand.,* **8**: 1235–1256.

Emerson, R., and D. L. Fox, 1940. Gamma-carotene in the sexual phase of the aquatic fungus *Allomyces. Proc. Roy. Soc. London, Ser. B,* **128**: 275–293.

Fox, D. L., 1948. Some biochemical aspects of marine carotenoids. *Fortschr. Chem. org. Naturstoffe,* **5**: 20–39.

Fox, D. L., 1953. Animal Biochromes and Structural Colors. Cambridge University Press, Cambridge.

Francis, D. W., 1964. Some studies on phototaxis of *Dictyostelium. J. Cell. Comp. Physiol.,* **64**: 131–138.

Goodwin, T. W., 1950. Carotenoids and reproduction. *Biol. Rev. Camb. Phil. Soc.,* **25**: 391–413.

Goodwin, T. W., 1952. Studies in carotenogenesis 3. Identification of the minor polyene components of the fungus *Phycomyces blakesleeanus* and a study of their synthesis under various cultural conditions. *Biochem. J.,* **50**: 550–558.

Goodwin, T. W., 1954. Carotenoids Their Comparative Biochemistry. Chemical Publishing Company, Inc., New York.

Goodwin, T. W., M. Jamikorn and J. S. Wilmer, 1953. Studies in carotenogenesis 7. Further observations concerning the action of diphenylamine in inhibiting the synthesis of beta-carotene in *Phycomyces blakesleeanus*. *Biochem. J.*, **53**: 531–538.

Haxo, F. T., 1949. Studies on the carotenoid pigments of *Neurospora*. I. Composition of the pigment. *Arch. Biochem.*, **20**: 400–421.

Haxo, F. T., 1955. Some biochemical aspects of fungal carotenoids. *Fortschr. Chem. org. Naturstoffe*, **12**: 169–197.

Jensen, S. L., 1958. The path of carotenoid synthesis in a photosynthetic bacterium. *Biochim. et Biophys. Acta*, **29**: 477–498.

Karrer, P., and E. Jucker, 1950. Carotenoids. Elsevier Publishing Company, New York.

Kharasch, M. S., 1936. Some chemical factors influencing growth and pigmentation of certain microorganisms. *J. Bact.*, **32**: 533–540.

Murneek, A. E., 1934. Relation of carotenoid pigments to sexual reproduction in plants. *Science*, **79**: 528.

Nash, H. A., 1945. Absorption spectrum of zeta-carotene. *Arch. Biochem.*, **7**: 305–312.

Nash, H. A., 1948. Studies on the structure of zeta-carotene. *J. Amer. Chem. Soc.*, **70**: 3613–3615.

Raper, K. B., 1937. Growth and development of *Dictyostelium discoideum* with different bacterial associates. *J. Agric. Res.*, **55**: 289–316.

Raper, K. B., 1939. Influence of culture conditions upon the growth and development of *Dictyostelium discoideum*. *J. Agric. Res.*, **58**: 157–198.

Raper, K. B., 1940. Pseudoplasmodium formation and organization in *Dictyostelium discoideum*. *J. Elisha Mitchell Sci. Soc.*, **56**: 241–282.

Turian, G., 1950. Recherches sur la biosynthese des carotenoids chez un Bacille paratuberculeux. III. Inhibition de la pigmentation par la diphenylamine. *Helv. Chim. Acta*, **33**: 1988–1993.

Turian, G., and F. Haxo, 1952. Further use of diphenylamine for the study of carotenoid biosynthesis in *Mycobacterium phlei*. *J. Bact.*, **63**: 690–691.

Whittingham, W. F., and K. B. Raper, 1956. Inhibition of normal pigment synthesis in spores of *Dictyostelium purpureum*. *Amer. J. Bot.*, **43**: 703–708.

Zalokar, M., 1957. Isolation of an acidic pigment in *Neurospora*. *Arch. Biochem. Biophys.*, **70**: 568–571.

Zechmeister, L., 1960. *Cis-trans* isomeric carotenoid pigments. *Fortschr. Chem. org. Naturstoffe*, **18**: 223–349.

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Volume 132 Number 1

THE
BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

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FEBRUARY, 1967

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Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

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THE BIOLOGICAL BULLETIN

THE BIOLOGICAL BULLETIN is issued six times a year at the Lancaster Press, Inc., Prince and Lemon Streets, Lancaster, Pennsylvania.

Subscriptions and similar matter should be addressed to The Biological Bulletin, Marine Biological Laboratory, Woods Hole, Massachusetts. Agent for Great Britain: Wheldon and Wesley, Limited, 2, 3 and 4 Arthur Street, New Oxford Street, London, W. C. 2. Single numbers, $3.75. Subscription per volume (three issues), $9.00, (this is $18.00 per year for six issues).

Communications relative to manuscripts should be sent to Dr. Donald P. Costello, Marine Biological Laboratory, Woods Hole, Massachusetts, between June 15 and September 1, and to Dr. Donald P. Costello, P. O. Box 429, Chapel Hill, North Carolina, during the remainder of the year.

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